

The use of filariae as a therapeutic agent for hypersensitivity diseases

by

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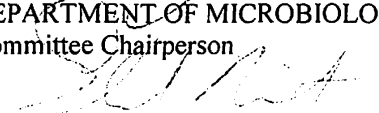

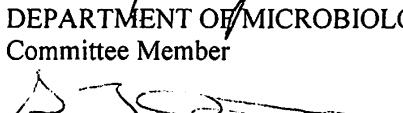
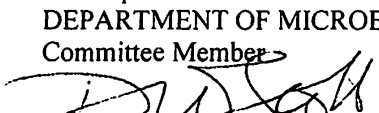
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PREFACE

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Finally, I would like to thank the mice for their contribution to this work.

DEDICATION

For my family – Thank you for encouraging me to pursue my dreams, wherever they may take me.

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ABSTRACT

The use of filariae as a therapeutic agent for hypersensitivity diseases:

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In this study we evaluated the effect chronic helminth infection has on allergic disease in mice previously sensitized to ovalbumin (OVA). Ten weeks of infection with *Litomosoides sigmodontis* reduced immunological markers of type I hypersensitivity, including OVA-specific IgE, basophil activation, and mast cell degranulation. Despite these reductions, there was no protection against immediate clinical hypersensitivity as measured by changes in vascular permeability following intradermal OVA challenge. However, late phase ear swelling, due to type III hypersensitivity, was significantly reduced in chronically infected animals. Immune complexes visualized in the ear were slightly larger in the infected group. Levels of total IgG2a, OVA-specific IgG2a, and OVA-specific IgG1 were all reduced in the setting of infection. These reductions were likely due to increased antibody catabolism as ELISPOT assays demonstrated that infected animals do not have suppressed antibody production. Ear histology 24 hours after challenge showed infected animals have reduced cellular infiltration in the ear, with significant decreases in numbers of neutrophils and macrophages. Consistent with this

finding, infected animals had significantly fewer neutrophil-specific chemokines CXCL1 and CXCL2 in the ear following challenge. Additionally, *in vitro* stimulation with immune-complexes resulted in significantly less CXCL1 and CXCL2 production by eosinophils from chronically infected mice. Expression of FcγRI was also significantly reduced on eosinophils from infected animals. Finally, we found that *L. sigmodontis* infection decreased anti-dsDNA autoantibodies and prevented mesangial expansion in mice with previously established systemic lupus erythematosus. These data indicate that chronic filarial infection suppresses eosinophilic responses to antibody-mediated activation and has the potential to be used as a therapeutic for pre-existing hypersensitivity diseases.

The development of an *in vitro* life cycle for filarial worms would allow for the mass production of worm antigen for use as an allergy or autoimmune therapeutic, as well as provide a platform for high throughput screening of novel macrofilaricides. Before an *in vitro* life cycle can be developed, worms need to be able to survive outside the mammalian and intermediate host for an extended period of time. In this study, we sought to characterize nutrients that promote the long-term survival of filarial worms *in vitro*. Using microfilariae (MF) obtained from gerbils infected with *L. sigmodontis*, a filarial parasite of rodents, we found that Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) resulted in an average survival of only 5 days. However, co-culturing MF with a mouse endothelial cell line (EOMA) enabled survival for 40 days. Culturing EOMA cells in transwell plates extended MF survival to the same degree as direct co-culture, suggesting that the factors microfilariae require are soluble in nature. Heat inactivation of EOMA-conditioned media at 56°C reduced MF

survival by approximately 50%, and heat inactivation at 100°C reduced survival to 3 days. This signifies that MF require both heat labile and heat stable factors. EOMA cells require FBS to produce these factors, as conditioned media collected from EOMA cells grown in the absence of FBS failed to prolong survival. The removal of lipids also abrogated survival, indicating MF need both protein and lipid factors. Dialysis experiments suggest that at least some of the required factors are between 100-1000 Da in size. Importantly, these findings also pertain to adult worms. Both rodent *L. sigmodontis* and human *Brugia malayi* adult worms also show significantly extended survival when cultured in EOMA conditioned media. Together, these results suggest that EOMA-produced factors include lipid-containing molecules, heat labile molecules (likely a protein), and micronutrients between 0.1 and 1 kDa in size. These studies are the first steps toward the characterization of essential nutrients filarial worms require for long-term survival *in vitro*.

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CHAPTER 1: Introduction

HELMINTH INFECTION AND HOST PROTECTION FROM ALLERGY

The prevalence of allergic disease is on the rise and new treatment options are needed. Despite promising epidemiologic and immunologic evidence suggesting helminth infections protect against allergic disease, human clinical trials have failed to show clinical benefit (15; 57). In order to explain these discrepancies, research endeavors will need to determine which types of allergic responses are best suppressed by helminth infection, and the mechanisms by which this suppression occurs. Furthermore, advances in the development of *in vitro* culture systems for helminths will allow for the characterization and mass production of helminth antigen(s) responsible for host protection from allergy.

ALLERGY

Allergy is a hypersensitivity disorder that occurs when the immune system responds to self-antigens or harmless environmental substances, such as pollen, food, drugs, latex, metals, insects, and plants. Both in the United States and globally, the prevalence of allergy has risen over the past several decades, with the majority of the burden affecting children (27; 28; 101; 171). Genetic and environmental factors, including exposure to allergens, infections, and pollutants, all play a role in determining whether an individual will develop allergic disease (108; 217). Allergies can be grouped into four major classes, referred to as types I-IV hypersensitivity. Each class results from a distinct mechanism of immunopathogenesis, though disease manifestations may vary depending on the tissue site affected.

Type I hypersensitivity

Type I hypersensitivity diseases include rhinitis, hay fever, anaphylactic shock, atopic dermatitis, conjunctivitis, food allergy, and some forms of asthma. Sensitization occurs when there is repeated exposure to low doses of allergen. This induces the release of thymic stromal lymphopoietin (TSLP) (128), IL-33 (105), and IL-25 (232) by epithelial cells that have come into contact with the allergen. These cytokines activate innate lymphoid cells (ILCs) and help dendritic cells (DCs) polarize a Th2 environment (113). A Th2-skewed immune response is characterized by the differentiation of Th2 CD4⁺ T cells, the generation of IL-4, IL-5, and IL-13, and B cell class switching to IgE production (112). IgE then binds to the high affinity FcεRI expressed on the surface of mast cells and basophils, the effector cells of type I hypersensitivity (Fig. 1A).

Upon subsequent exposure, the allergen induces cross-linking of IgE on the surface of mast cells and basophils. This results in cellular degranulation and the release of vasoactive and pro-inflammatory mediators such as histamine, tryptase, and platelet-activating factor (PAF) (166). Following the release of pre-formed mediators during degranulation, mast cells synthesize and release prostaglandins and leukotrienes, which further increase vascular permeability (10).

The initial clinical response occurs rapidly and is referred to as an “immediate reaction.” In the skin, a wheal-and-flare response is readily visible within minutes of allergen challenge. Depending on the dose of the challenge, it is possible to observe a “late phase response” hours later. Late phase swelling is due to localized edema and leukocyte recruitment into the tissues (166). Histamine, generally considered to be the major mediator in immediate allergic responses, acts on histamine receptor 1 (HR1) expressed on vessels and histamine receptor 2 (HR2) expressed on smooth muscle. The

combined effect increases blood flow and vascular permeability at the site of inflammation (158) (Figure 1B).

Type II hypersensitivity

Extrinsic type II hypersensitivity is due to antibody-mediated destruction of cells, which often occurs in the setting of drug allergies. Drugs such as penicillin and quinolones are classified as haptens: small molecules that have the capacity to induce an immune response by binding to soluble or membrane-bound proteins (176). The immune system recognizes hapten-protein complexes as foreign antigenic structures, and IgG antibodies are generated. IgG binding and complement activation induce a cytotoxic mechanism of cell clearance by the liver or spleen, which can result in hemolytic anemia or thrombocytopenia (176).

Intrinsic type II hypersensitivity is due to the production of autoantibodies. For rheumatic fever, molecular mimicry causes antibodies generated against *Streptococcus pyogenes* to cross-react with host tissue. Cross-reactivity can occur to brain, joint, skin, and heart tissue, leading to cell death and inflammation (41). Myasthenia gravis and Grave's disease are additional autoimmune disorders characterized as type II hypersensitivities. Autoantibodies developed against acetylcholine receptors at neuromuscular junctions leads to muscle weakness in myasthenia gravis (118), and production of autoantibodies directed against thyrotropin receptors triggers hyperthyroidism and goiter in Grave's disease (233).

Figure 1. Type I hypersensitivity

(A) Sensitization stage. Sensitization occurs after repeated exposure to low doses of an allergen. Epithelial cells release TSLP, IL-25 and IL-33. These cytokines activate DCs, basophils, and mast cells to induce a Th2-skewed immune environment. Naïve T cells differentiate into Th2 cells and secrete Th2-specific cytokines IL-4, IL-5, and IL-13. These cytokines induce eosinophilia, B cell class switching to IgE synthesis, and stimulate mucus production. Reprinted with permission from Elsevier Science Ltd: Immunity, copyright 2009 (112). **(B)** Effector stage. Subsequent exposure to an allergen results in the cross linking of IgE on the surface of mast cells and basophils. Cellular degranulation releases histamine and other pro-inflammatory mediators. Immediate hypersensitivity occurs within minutes of allergen exposure and results from increased vascular permeability. The late phase response occurs hours later due to leukocyte recruitment to the tissue, and can result in localized swelling or bronchoconstriction of the airways, depending on the route of allergen exposure. Reprinted with permission from Macmillan Publishers Ltd: Nature Medicine, copyright 2012 (67).

The diagram illustrates the role of DCs in allergic sensitization. At the top, allergens (orange spiky particles) are shown binding to the surface of a row of DCs (green) and a mast cell/basophil (red). The DCs are labeled with 'Allergens' and 'TSLP, GM-CSF, IL-33, IL-1'. The mast cell/basophil is labeled with 'TSLP, IL-33'. Arrows indicate the release of these factors. The DCs then interact with a 'Naive' Th2 cell (blue) via 'OX40-L' and 'IL-4 TSLP?'. The Naive Th2 cell is labeled with 'CCL17, CCL22, Eotaxin'. The mast cell/basophil releases 'IL-5, IL-13, GM-CSF, IL-6'. The Naive Th2 cell produces 'CCL20, IL-25'. The final outcome is 'IgE production, eosinophilia, mucus production, bronchial hyperreactivity'.

This diagram illustrates the immediate hypersensitivity reaction occurring minutes after antigen exposure. The process begins with an antigen (Ag) interacting with a dendritic cell (DC) and a basophil. The basophil, which has upregulated its surface FcεRI receptors, is activated by the antigen. This activation leads to the release of histamine, PGD₂, LTC₄, and TNF, which causes increased mucus production from goblet cells. The released mediators also cause vasodilation and increased vascular permeability, leading to plasma proteins and fluid entering the tissue. This results in epithelial injury and cellular death. The reaction is characterized by immediate hypersensitivity (minutes after Ag exposure) and a late phase reaction (hours after Ag exposure) involving leukocyte recruitment and activation. The final outcome is reduced airflow and wheezing due to bronchoconstriction and airway smooth muscle cells. A note indicates that the upregulation of surface FcεRI and enhanced survival are specific to mice.

Type III hypersensitivity

Type III hypersensitivity was first discovered by Maurice Arthus in 1903 when he gave repeated subcutaneous injections of horse serum to rabbits and observed swelling, edema, and necrosis at the site of injection (12). Later known as an “Arthus reaction” or “Arthus phenomenon” (125), type III hypersensitivity was discovered to be driven by the formation of immune complexes (36).

Type III hypersensitivity is similar to other allergic responses in that sensitization with an antigen results in the production of antigen-specific antibodies. Immune complexes form between the antibodies and the antigen upon subsequent exposure. IgG2a in mice (IgG1 in humans) is the primary antibody isotype participating in immune complex formation (165). While IgG2a is considered to be pro-inflammatory, anti-inflammatory IgG1 antibodies in mice (IgG4 in humans) can also be present in the immune complex (165). Once immune complexes form, complement is activated and an inflammatory cascade is initiated. If antigen is injected intravenously, soluble circulating immune complexes deposit on vessel walls. When antigen is injected into the skin, immune complexes form in the tissue and necrotic lesions develop (189) (Fig. 2).

Receptor binding

The Fc regions of antibodies present in immune complexes bind to Fc gamma receptors (FcγRs) on the surface of leukocytes. There are three activating FcγRs in mice: FcγRI, FcγRIII, and FcγRIV (24). FcγRI is a high-affinity receptor and FcγRIII and FcγRIV are low-affinity receptors. All signal through immunoreceptor tyrosine-based action motif (ITAM) domains. Upon aggregation of activating FcγRs, ITAM domains are phosphorylated and a signaling cascade activates NF-κB and other nuclear transcription

factors (42). Depending on the cell type, activating FcγRs can trigger antibody-dependent cell-mediated cytotoxicity (ADCC), cell degranulation, or the production of pro-inflammatory cytokines and chemokines (42). Finally, signaling through ITAMS can result in endocytosis of the ligand (42).

FcγRIIb is the only inhibitory receptor and signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (42). Phosphorylation of the ITIM domain ultimately leads to dephosphorylation and inhibition of signaling cascade targets induced by activating FcγRs (205). Thus, the ratio of activating and inhibitory FcγRs stimulated by immune complexes determines whether a pro- or anti-inflammatory response is initiated. FcγRIIb is especially important for the regulation of immune responses. Polymorphisms in the FcγRIIb gene have been linked to autoimmune diseases, defects in pathogen clearance, and immunopathogenesis associated with infectious agents (205).

Cell recruitment

In the setting of type III inflammation, immune complexes bind FcγRI and FcγRIII on resident macrophages and mast cells. These cells then produce pro-inflammatory mediators and chemokines to recruit neutrophils and monocytes (103). CXCL1 (KC) and CXCL2 (MIP-2) are closely related neutrophil chemoattractants (242). KC is considered to be a murine ortholog for IL-8 whereas MIP-2 has sequence homology to Groα/MGSA (22). While both chemokines bind to the G protein-coupled receptor CXCR2 to facilitate neutrophil chemotaxis and migration (23; 183), MIP-2 is considered the primary driver of neutrophil recruitment. Neutralizing antibodies against MIP-2, but not KC, prevents antigen-induced neutrophil recruitment, suggesting a redundant role for KC in inflammation (183). MIP-2 triggers a dose-dependent

accumulation of neutrophils by inducing the production of MIP-1 α , TNF- α , and LTB₄ in a sequential fashion (182). In addition to its chemoattractant properties, MIP-2 has also been shown to stimulate the degranulation of neutrophils and release of lysozyme (242).

Neutrophils are particularly important for inducing inflammation, as the severity of an Arthus reaction can be directly correlated to the number of neutrophils recruited to the tissue (103). Neutrophils transition into an activated state following transmigration through the endothelium (80), and recognize immune complexes through ligation of complement and Fc gamma receptors. Neutrophil degranulation and release of proteases (179) and reactive oxygen species (190) induces apoptosis and necrosis of the surrounding tissue. Pathology is controlled through the production of lipid mediators, IL-4, and IL-10 by resident cells (155) and IL-1RA by neutrophils (200).

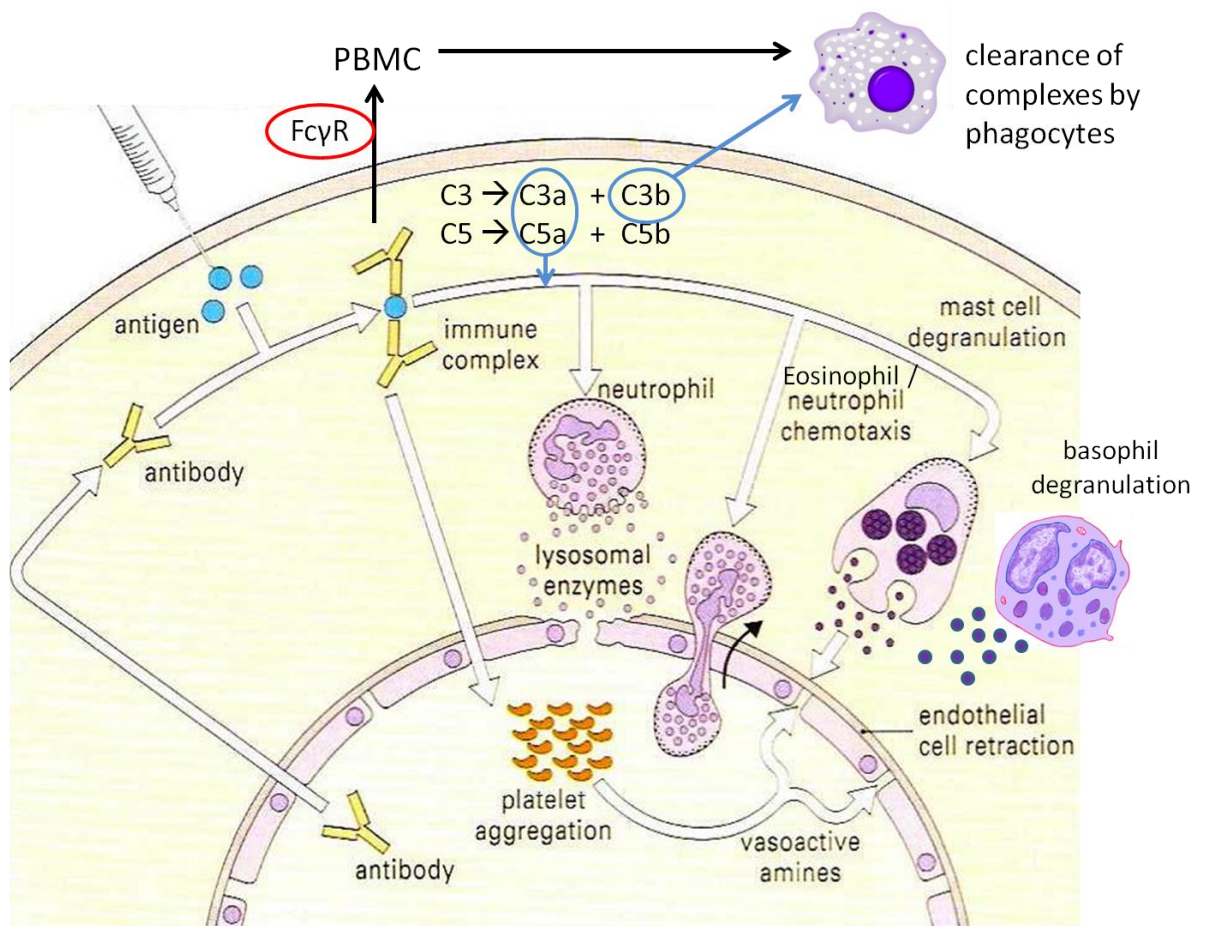
Apart from contributing to the formation of IgG immune complexes, antigen may also bind to IgE if it is present on the surface of mast cells. The activation of mast cells in an IgE-dependent manner induces the release of eosinophil-specific chemokine MCP-3 (93). Recruited eosinophils have the capacity to enhance inflammation through the production of additional neutrophil-specific chemokines (191).

Systemic lupus erythematosus

In addition to the Arthus reaction, which can sometimes occur during vaccination (203), serum sickness (110), post-streptococcal glomerulonephritis (63), and systemic lupus erythematosus (SLE) (177) are all clinical diseases caused by type III hypersensitivity reactions. SLE is a highly heterogeneous disease arising from the

Figure 2. Type III hypersensitivity

Antibodies bind to antigen to form immune complexes. Immune complexes activate complement and ligate receptors on the surface of resident cells to induce the production of neutrophil and eosinophil-specific chemokines. Cells are recruited to the tissue and degranulate upon immune complex stimulation, further exacerbating inflammation. Complexes are eventually cleared from the tissue by phagocytes. Figure adapted from (189).



production of autoantibodies against double-stranded DNA (dsDNA) and nuclear proteins. The etiology of SLE is complicated, with over 140 genes implicated in the development of the disease (187). Abnormalities in the clearance of apoptotic and necrotic cells lead to a breakdown of tolerance (84), allowing for autoantibodies to react with dsDNA and nuclear proteins to form immune complexes.

The Systemic Lupus International Collaborating Clinics (SLICC) revised the SLE criteria developed by the American College of Rheumatology (ACR) in 1982 (218). Patients must demonstrate 4 out of the 17 clinical and immunologic criteria to be diagnosed as having SLE (174). Clinical criteria include a malar/photosensitive rash, arthritis, hemolytic anemia, nephritis, and neurologic manifestations (174). Immunologic criteria include anti-double-stranded DNA, anti-Sm, anti-nuclear antibodies, low complement, and lymphopenia (174). The diverse range of clinical symptoms expressed by SLE patients is representative of the various tissue types that can be affected by immune complex-mediated tissue damage.

Type IV hypersensitivity

Rashes arising from exposure to poison ivy (225), contact dermatitis due to nickel hypersensitivity (225), and the Tuberculin skin test (20) are all examples of type IV hypersensitivity. Also known as cell-mediated or delayed type hypersensitivity, this class of allergic inflammation is caused by T cells. Antigens expressed on MHC class II are recognized by memory Th1 CD4⁺ T cells, which in turn produce the pro-inflammatory cytokines TNF- α and IFN- γ (72). Infiltrating cytotoxic CD8⁺ T cells recognize antigen presented on MHC class I and induce host cell death (72). The combined effect of CD4⁺ and CD8⁺ T cell activation results in swelling, pain, and erythema (225).

Animal models of allergy

In this study, we focus on evaluating the effects of helminth infection has on type I and type III hypersensitivity.

Type I hypersensitivity

A variety of animal models of allergy have been developed to study the mechanisms involved in allergic inflammation. Ovalbumin (OVA), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), and peanut extract are all allergens that are commonly used to induce type I hypersensitivity (52). Intraperitoneal (i.p.) injections of the allergen with an adjuvant result in systemic sensitization, whereas intratracheal or intranasal administration leads to asthmatic sensitization (154), and oral gavage induces food allergy (39).

The site of allergen challenge can also be varied to elicit inflammation in diverse tissue types. Cutaneous hypersensitivity reactions can be achieved via subcutaneous (s.c.) injection of the allergen after sensitization. Local anaphylaxis measured by dye extravasation (54), late phase ear swelling (152), and delayed type hypersensitivity are all outcomes that can be assessed by this technique. Intratracheal or intranasal challenge is a classic method used to induce airway hyperresponsiveness (AHR) for the study of asthmatic inflammation (154).

In addition to monitoring clinical responses, immunological markers of allergic disease are often measured to assess disease severity and the efficacy of therapeutic agents. Mast cell degranulation can be used as a marker for cutaneous, IgE-mediated allergic responses (77). Other parameters include levels of total and allergen-specific

IgE, basophil activation, eosinophil recruitment, and Th2 cytokine production (IL-4, IL-5 and IL-13) (133).

Type III hypersensitivity

Type III hypersensitivity can also be initiated via i.p. sensitization and s.c. challenge. Because the animal is sensitized systemically, both IgE and IgG are developed against the allergen, which allows for the formation of immune complexes upon subsequent exposure. Timing is critical for distinguishing an Arthus reaction from type I and type IV hypersensitivities. Whereas local anaphylaxis occurs within minutes of allergen exposure and delayed type hypersensitivity takes several days to develop, type III hypersensitivity responses peak between 12-24 hours after challenge (156).

Animal models of SLE are based on chemical induction or spontaneous development of the disease. Pristane, a hydrocarbon oil adjuvant, stimulates the production of anti-dsDNA, anti-nRNP, and anti-Sm autoantibodies in BALB/c and C57BL/6 mice. Administration of pristane typically results in 80% penetrance, and can be used as a model for SLE-associated arthritis, glomerulonephritis, and pulmonary disease (124). Spontaneous lupus arises in BSXB/*Yaa* and MRL/*lpr* mouse strains, as well as from F1 hybrids between New Zealand White (NZW) and New Zealand Black (NZB) strains. These strains have predominantly been used to shed light on the genetic factors contributing to the development of SLE (172).

Treatments for allergic disease

Common treatment options for type I hypersensitivity include antihistamines, monoclonal antibodies, and allergen-specific immunotherapy (SIT). Antihistamines are medications that are readily available for over-the-counter and prescription use. The

mechanism of action for antihistamines is the blockade of H₁, which prevents the inflammation and cell activation associated with histamine release (35).

The monoclonal antibody omalizumab has been shown to reduce symptoms for patients suffering from allergic rhinitis (49), skin reactivity, and asthma (198).

Omalizumab targets the Fc portion of IgE, blocking it from binding to FcεRI on the surface of mast cells and basophils, and reducing the amount of circulating IgE (198). Clinical benefit arises from reduced FcεRI expression and sensitivity to IgE cross-linking by basophils (49). Omalizumab has also been administered concurrently with rapid oral desensitization to peanuts, improving both the safety and efficacy of allergen SIT (195).

Allergen SIT desensitizes an allergic individual through repeat administration of low doses of the allergen. Allergen SIT is often used for seasonal or pet allergens, and typically takes place over long periods of time with patients receiving monthly or biweekly injections. In addition to lessening symptoms, allergen SIT stops pre-existing allergies from increasing in severity and prevents sensitization to new allergens (5).

Immune tolerance resulting from immunotherapy is characterized by decreased levels of circulating IgE and a corresponding increase in IgG4 (73), the induction of allergen-specific T regulatory cells, and production of immunosuppressive cytokines IL-10 and TGF-β (62; 104).

Rush desensitization can be completed over the course of several hours in a hospital setting, and allows a patient to receive a necessary drug or antibiotic to which they are allergic. The immunologic mechanisms responsible for rush desensitization are currently unknown, however the internalization of FcεRI on the surface of mast cells is one potential mechanism (163). Nevertheless, long-term tolerance is not achieved

through rush desensitization as patients regain hypersensitivity to the drug once rush treatment stops.

While type I and type IV hypersensitivities are good candidates for allergen-specific immunotherapy, this method of treatment is contraindicated for type II and type III hypersensitivities (91). Drug avoidance is the recommended course of action for extrinsic type II hypersensitivities (91). For intrinsic type II hypersensitivities, treatment is centered on restoration of normal physiologic function of affected tissues. For example, radioactive iodine therapy, anti-thyroid medications and surgery combat the hypothyroidism associated with Grave's disease (153). Treatment of type III hypersensitivities, especially SLE, is difficult given the variety of tissue types that can be affected. Treatment options are limited to non-steroidal anti-inflammatory drugs (NSAIDS), antimalarials, intravenous immunoglobulin (IVIG), steroids, and other immunosuppressants, many of which cause adverse side effects (243).

Overall, there is an overwhelming need for the development of new therapeutic strategies for hypersensitivity diseases. Current treatments for allergic disease are palliative rather than curative, with allergen avoidance typically providing the best results.

THE HYGIENE HYPOTHESIS

The hygiene hypothesis was developed to explain the high prevalence of allergy and autoimmune disorders in industrialized countries (1; 211). The hypothesis proposes that an absence of childhood infections can lead to immune dysregulation and allergic disease (211; 212). Although the original study investigated the effects of household size

on hay fever and eczema (211), subsequent studies have linked infectious agents, especially parasitic infections (74; 227), with protection against atopy.

Allergy and helminths: Epidemiology

Helminths are a polyphyletic group of parasitic worms composed of cestode, trematode, and nematode taxa. Helminths occupy diverse niches in the body and can induce a wide variety of diseases. Helminth infections are designated as Neglected Tropical Diseases (NTDs) by the World Health Organization (WHO) due to vast geographical distribution, high prevalence, and lack of available funding for treatment and research programs (236; 237).

Epidemiological studies have shown an inverse relationship between helminth infections and allergic disease (37; 146; 216; 227; 230). The helminth species implicated in protection are varied, and include schistosome, filarial, and intestinal worms. Helminths primarily protect against asthma (146) and allergic skin reactivity (37; 216; 227; 230). It is difficult to ascertain whether active infection is required for allergy protection, as there is conflicting data regarding the effects of anthelmintic treatment on atopy. Several groups showed that anthelmintic treatment increases the risk of atopy (61; 226), yet others reported no effect (38; 192) or a clinical benefit (134).

Immune responses generated against helminth infections

Although helminths induce Th2 responses, chronic infections rarely generate symptoms associated with allergic disease (26; 60; 206). Similar to sensitization with an allergen, worm-derived pathogen associated molecular patterns (PAMPs) are able to stimulate pattern recognition receptors (PRRs) on epithelial cells, mast cells, basophils, and DCs to induce the release of TSLP, IL-25 and IL-33 (94). These mediators then

activate ILCs to produce Th2 cytokines IL-4, IL-5 and IL-13, and a type-2 immune response is initiated.

It has been shown that strong type-2 and ILC responses tend to confer resistance to infection in animal models (58). IL-13 is also particularly important for worm clearance. IL-13 stimulates mucus secretion by goblet cells and smooth muscle contraction to expel worms from the gastrointestinal tract and lungs (140; 151). Worm expulsion is also enhanced by increased epithelial cell turnover following IL-13 and IL-25 release (94).

High levels of IL-5 drive eosinophilia, a hallmark of helminth infection. Eosinophils help mediate clearance of infection by killing worms via ADCC (111). IL-4 induces B cell class switching and the production of worm-specific and non-specific IgE and IgG (11). These antibodies are able to bind to the surface of the worm, and FcεRI in the case of IgE, allowing for granulocyte killing of the parasite through the release of cytotoxic granules (111).

In addition to clearing parasitic infections, a Th2 immune environment promotes wound healing. This is necessary in the context of helminth infection as worms have invasive, tissue-migratory phases that elicit significant damage to host tissue. Th2 cytokines induce the alternative activation of macrophages, which dampens Th1 and Th17 responses and the inflammation associated with tissue damage (11). Alternatively activated macrophages release arginase and IGF-1, both of which contribute to wound healing by inducing collagen synthesis and cell proliferation (30). Neutrophil influx is also decreased due to the presence of IL-4 and IL-10, and the suppression of IL-17 production (30).

Allergy and helminths: Animal models

Infection with live helminths

A substantial amount of work has been performed with animal models to explore helminth infection and host protection from allergy, with more than 30 such studies published to date (55). However, instead of testing whether helminth infections can treat established allergy, the majority of research has addressed whether helminths protect the host against the development of allergy; that is, in most experiments animals were infected first and then sensitized to an allergen. The majority of these experiments, which were conducted with a wide range of hypersensitivity models, demonstrated that helminths protect against development of allergic disease (55).

Surprisingly, only four groups have performed experiments to determine whether helminth infections are capable of protecting the host from established allergy (96; 160; 239; 241). Of these studies, only two showed protection (160; 239). Infection with *N. brasiliensis*, an acute model of hookworm infection, did not protect against AHR, local anaphylaxis, or systemic anaphylaxis (96; 241). However, *H. polygyrus* and *S. venezuelensis* were two species of parasites that elicited protection against AHR-mediated allergic inflammation (55; 160; 239).

Use of helminth antigen

The use of worm antigen as a therapeutic for established allergy may be a useful alternative to natural infection because it bypasses some of the potentially harmful side effects associated with live worm infections. However, there are also potential disadvantages to using worm antigens. These include expenses incurred for antigen production and purification, the probable need for frequent administration, and the risk of

allergic reactions to the worm antigens themselves. Despite these potential pitfalls, studies with worm antigens have shown promising results in animal models (55).

Protection from allergy using worm antigens has been tested in numerous studies, with the majority of the work again dedicated to protecting the host from developing allergic disease. Overwhelming evidence has shown that specific antigens (25; 168; 196), pseudocoelomic fluids (142; 170), excretory-secretory products (143; 145; 224), whole-worm antigen preparations (100; 120; 126), and egg antigen (244) are all capable of protecting the host against the development of allergy (55).

Three groups have shown that administration of parasite antigens after sensitization is capable of reducing the symptoms associated with established allergy. Intraperitoneal administration of the *Anisakis simplex* ortholog of macrophage migration inhibitory factor (168), *Clonorchis sinensis* total worm antigen (100), and *Acanthocheilonema viteae* cystatin (196) protected animals from allergic responses when using an AHR model of allergic inflammation (55).

Immune modulation by helminths

One of the interesting aspects of chronic helminth infections is that they enhance immune regulatory mechanisms over time. Helminth-induced immunomodulation is multifactorial and causes suppression of both the innate and adaptive arms of the immune system (14; 144). There are multiple mechanisms by which worms are able to overcome host resistance to infection, including the induction of *de novo* forkhead box protein 3 expression on T cells (78) and IL-6 production (204). Alterations in dendritic cell function, increased production of downregulatory cytokines (such as IL-10 and TGF- β) and increased frequencies of regulatory T and B cells have all been implicated as

mechanisms underlying immune hyporesponsiveness. In addition to establishing systemic immunoregulatory networks, helminths secrete a wide variety of immunomodulatory molecules, including glycoprotein ES-62 (76), cysteine protease inhibitors (82; 175), and orthologs of host cytokines (75; 228). Immune suppression is likely due to the direct effect of worm products on immune cells, as well as chronic activation of the immune system with subsequent induction of negative feedback mechanisms.

AHR has been the most common type of allergy model used in animal studies that combine helminth infection and allergic disease. In most of these studies, helminths appear to protect against allergy by inhibiting the infiltration of inflammatory cells, especially eosinophils, into the airways. The majority of publications have linked reduced airway pathology to decreased levels of chemokines (including eotaxin 1) and type 2 cytokines (including IL-4, IL-5, and IL-13) in the bronchoalveolar lavage fluid. Helminth excretory-secretory products have also been shown to block IL-33 release and suppress ILC responses, which results in suppression of allergic airway disease (143).

Although the transfer of cells from infected animals into sensitized naive animals has been successful at protecting the host from allergic inflammation, there is not yet a clear consensus on which cell type is most important for eliciting protection. At this time, cells that actively downregulate immune responses, including regulatory T and B cells, are the top contenders for inducing protection (55).

In studies that combined helminth infection with an allergy model, a reduction in allergen-specific antibody titers in the infected host was often observed. This phenomenon occurred with numerous species of helminths, including *H. polygyrus* (17; 180), *L. sigmodontis* (47), *Schistosoma mansoni* (164), *Schistosoma japonicum* (127;

149), *Trichinella spiralis* (167), and *Strongyloides stercoralis* (231). Furthermore, immunization with parasite antigens in lieu of a natural infection also decreased allergen-specific antibody titers (25; 100; 126; 145; 196; 224). The mechanism through which this occurs is not fully understood; however, clearance of IgE antibodies through CD23 (31) might be a plausible explanation that warrants further exploration. Although reduction in allergen-specific IgE levels does not always correlate with clinical benefit, it often occurs during allergen SIT (5), suggesting these data might prove to be an important base on which to frame future research endeavors.

One of the most longstanding hypotheses for the mechanism by which helminths protect against allergy has been the IgE blocking hypothesis (1), which states that high levels of IgE induced by helminth infections inhibit allergic sensitization by saturating IgE receptors. Although this is biologically plausible, a number of animal and *in vitro* studies suggest this phenomenon plays only a minor, if any, role in helminth-mediated protection against allergy (148; 178; 241). Furthermore, a study that surveyed different populations living in Saskatchewan also found no correlation between serum IgE levels and atopic disease in human subjects, signifying that IgE levels are likely due to genetic or environmental factors and might not be good predictors of allergic disease (69).

The extent to which the suppression of allergy effector cells is important for helminth-induced protection against allergy remains unknown. Recently, our group demonstrated that chronic helminth infection suppresses basophil activation in response to IgE receptor cross-linking (114). However, functionality of mast cells and basophils in a combined allergy/helminth model has yet to be studied (55).

Human clinical trials

On the basis of epidemiologic studies and the successful use of helminths to prevent allergy in animal models, two clinical trials tested whether helminths could protect against allergic disease in humans. These trials include the use of *Trichuris suis* to treat allergic rhinitis and *Necator americanus* to treat asthma.

Trichuris suis for allergic rhinitis

Administration of *Trichuris suis* ova (TSO) has been demonstrated to improve symptoms of patients with ulcerative colitis and Crohn's disease (214; 215).

Investigators sought to expand on these findings by testing whether TSO would also be helpful for patients with allergic rhinitis. Performed in Denmark, the study recruited patients with allergic rhinitis during the prior 2 pollen seasons that had grass-specific IgE and positive skin prick test responses for grass pollen. Modeled after the protective Crohn's disease and ulcerative colitis trials, participants in the double-blind, randomized, placebo-controlled study received either 2500 TSO or placebo every 21 days for a total of 24 weeks. Patients were exposed to helminths for at least 1 month before the peak of the pollen season (15).

Measures of mean daily symptom scores demonstrated that treatment with TSO did not reduce allergic rhinitis symptoms during the pollen season. Additionally, skin prick test response positivity, total histamine levels, and grass pollen IgE levels did not change as a result of TSO treatment. The only improvement observed was a reduction in the use of tablet medication during the study, although the use of nasal spray and eye drops remained the same (15).

***Necator americanus* for asthma**

In a nested case-control study carried out in Jimma, Ethiopia, investigators found that hookworm infection with a parasite burden resulting in more than 50 eggs per gram of feces was associated with a reduced risk of wheeze (199). A double-blind, randomized, placebo-controlled clinical trial was then performed to determine whether *N. americanus* infection could reduce the symptoms of asthma (57). The study recruited subjects given a diagnosis of clinical asthma by a physician who were currently taking daily inhaled corticosteroids. After exposure to 10 infectious larvae or histamine as a control, groups were asked to keep a daily diary for symptoms and were monitored for 16 weeks. For the primary outcome of the study, bronchial hyperresponsiveness was measured through sequential inhalations of AMP at 2-minute intervals until forced expiratory volume decreased by at least 20% (PD20AMP) or maximum AMP had been inhaled. At the end of the study, there was no difference in PD20AMP values between infected and uninfected subjects, nor were there improvements in self-reported asthma symptoms, the secondary outcome (57).

Mechanisms of protection

When taking both human and animal studies into perspective, there are several key points to consider in determining why clinical trials using helminth infections have been unsuccessful at treating allergic disease. The effects helminths have on allergic disease are ultimately caused by changes in host immune function. Thus, it is likely that immunologic changes need to occur at the site of the allergic reaction for protection to take place. In one of the two animal studies that showed a therapeutic benefit of live worms, the helminth used (*S. venezuelensis*) migrated through the same tissues as those

involved in the allergic disease being evaluated (lungs in an AHR model) (160).

Although the helminth used in the other successful therapeutic study of live worms in mice did not reside in the same tissue space as the allergic disease (*H. polygyrus*, an intestinal nematode, protected against AHR), the investigators did find that the infection induced systemic immunologic changes (239). Similarly, all three of the protective helminth antigen studies administered antigen systemically. In contrast to these studies, the negative clinical trial for allergic rhinitis used *T. suis*, a relatively noninvasive nematode that localizes to the intestinal tract. *T. suis* might not have protected against allergic rhinitis because it could not induce immunologic changes at the nasal mucosa.

Chronicity of infection may also be important for eliciting protection. Several studies have demonstrated that acute infection had either no effect or exacerbated allergic disease, whereas chronic infection was protective (117; 197; 207). This argues that chronic infection may be necessary to observe protection against allergic inflammation. Additionally, worm burden may be another important parameter. For safety reasons, only 10 *N. americanus* larvae were given to each study participant, however protective animal studies with live worms administered much higher doses of infectious larvae. Specifically, mice were given 5000 *S. venezuelensis* L3 larvae or 200 *H. polygyrus* L3 larvae to show a protective effect in models of AHR. Given the enormous difference in size between human subjects and mice, for 10 *N. americanus* larvae to be effective in human subjects, one would expect that a dose of only 1 or 2 larvae would be effective in mice. To date, studies with such low numbers of worms in animal models have not been conducted.

Interestingly, animal studies demonstrating the successful use of helminths as therapeutic agents were primarily performed in airway hypersensitivity models. In contrast, studies that did not find protection were conducted in cutaneous and systemic anaphylaxis models. The latter models of allergy are predominantly dependent on classical IgE-mediated type 1 hypersensitivity, whereas the pathogenesis of disease in airway hypersensitivity is multi-factorial. When considering asthma, it is important to note that our understanding of its cause is currently evolving. Not all forms of asthma are considered to be classic eosinophil-mediated responses (131; 157). Thus, when investigating the use of worms or worm products, precautions should be taken to note the mechanistic endotype of asthma included in the study. Finally, although there is evidence that infection can protect against type I and type IV hypersensitivity, no study has addressed whether helminths have a therapeutic effect on type III hypersensitivity.

It is unlikely that a silver bullet exists when it comes to using helminth infections to treat allergy. It is therefore worthwhile to consider the idea that particular species of helminths, or specific antigens, are best suited for particular types of allergic diseases. Overall, the type of infection (localized vs. systemic), intensity of infection (light vs. heavy), duration of infection (acute vs. chronic), type of allergic response (asthma vs. atopy), use of concomitant medications, host genetics, and species of helminth might all play a role in determining whether the host will be protected from allergy (55).

LYMPHATIC FILARIASIS

Filariae are tissue-invasive helminths that are transmitted by arthropod vectors. Filarial worms are the causative agents of onchocerciasis, loiasis, and lymphatic filariasis (LF). LF is a debilitating disease that causes significant morbidity and economic loss

(34). The species of parasites responsible for LF include *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. Because filarial worms cause chronic, systemic infection, induce strong immunoregulation, and can infect rodents, they are among the top models of helminth infection used for studying host protection from allergy.

Epidemiology

The WHO estimates that approximately 120 million individuals around the world have LF with another one billion at risk for contracting the disease; however these numbers are now likely smaller due to the establishment of an elimination program by the WHO in the year 2000. LF predominantly exists in sub-Saharan Africa and South East Asia, and is classified as an NTD (236).

Wuchereria bancrofti

W. bancrofti infection accounts for the majority of LF disease burden and approximately 90% of all LF cases (236). Transmission of *W. bancrofti* takes place in Sub-Saharan Africa, Asia, and parts of Central and South America (45). *W. bancrofti* was thought to have gained entry to the Americas through the slave trade (115), and existed in the United States until the 1930s (32). Depending on the region, *W. bancrofti* can be transmitted by either *Anopheles*, *Culex*, or *Aedes* species of mosquitoes (21). There are no animal reservoirs, indicating there is potential for the eradication of *W. bancrofti* (2; 21).

Brugia malayi

In contrast to *W. bancrofti*, *Brugia malayi* infects several mammalian reservoirs, such as cats, dogs, and monkeys. This makes control of LF in *Brugia*-endemic regions difficult, as elimination of environmental sources of the parasite is not feasible. *B. malayi*

is transmitted by *Anopheles*, *Aedes*, and *Mansoni* species of mosquitos. Transmission occurs in Asia and Southeast Asia, with foci typically found within close geographical proximity to freshwater swamps (50).

Brugia timori

B. timori is closely related to *B. malayi*, however it has evolved to occupy Timor and select islands off the eastern coast of Indonesia; *B. malayi* is not present in regions endemic for *B. timori*. The only known vector for *B. timori* is the Anopheline mosquito *A. barbirostris* which breeds in irrigated rice paddies. Unlike *B. malayi*, no animal reservoirs exist for *B. timori* (59).

Life cycle

The life cycle for parasites that cause LF begins with the transmission of infectious L3 larvae via the bite of an infected mosquito (Fig. 3). The L3 larvae are not injected into the skin during feeding, but rather drop from the mouth parts and enter the skin through the puncture wound created by the proboscis (2). L3 larvae migrate through the subcutaneous tissue and quickly transit to the lymphatic system. L3 larvae then molt to the L4 and L5 (adult) stage within the lymphatics. Adult larvae tend to prefer lymphatics located in the lower extremities, such as near the inguinal lymph nodes (45). However, worms can be recovered throughout the body, including in lymphatics draining from breast tissue. Adult worms typically live within the host for 5-7 years, however there have been reports of worms surviving as long as 40 years (45).

Adult worms are diecious and must find a partner of the opposite sex to mate. Patent infection is established when female worms are inseminated and produce microfilariae (MF), with each female capable of producing as many as 10,000 MF per

day (45). MF in the blood stream are taken up by mosquitoes during subsequent blood meals. Microfilaremia often exhibits periodicity, where MF are present in the blood at the same time of day that the vector takes blood meals. For example, Anopheline mosquitoes are the primary LF vectors in Africa. *W. bancrofti* MF display nocturnal periodicity, with peak microfilaremia occurring between 10 pm – 3 am, as to enhance the chances of being ingested during a blood meal (83).

Once inside the vector, MF penetrate the midgut and migrate to the flight wing muscles (53). They then enter a muscle cell and advance to the next larval stage (L2) intracellularly. L2 larvae are called “sausage stage” because at this developmental phase they decrease in length and increase in girth. They molt again to become L3 stage, exit the musculature, and travel to the head of the mosquito for transmission during the next blood meal (2).

Disease

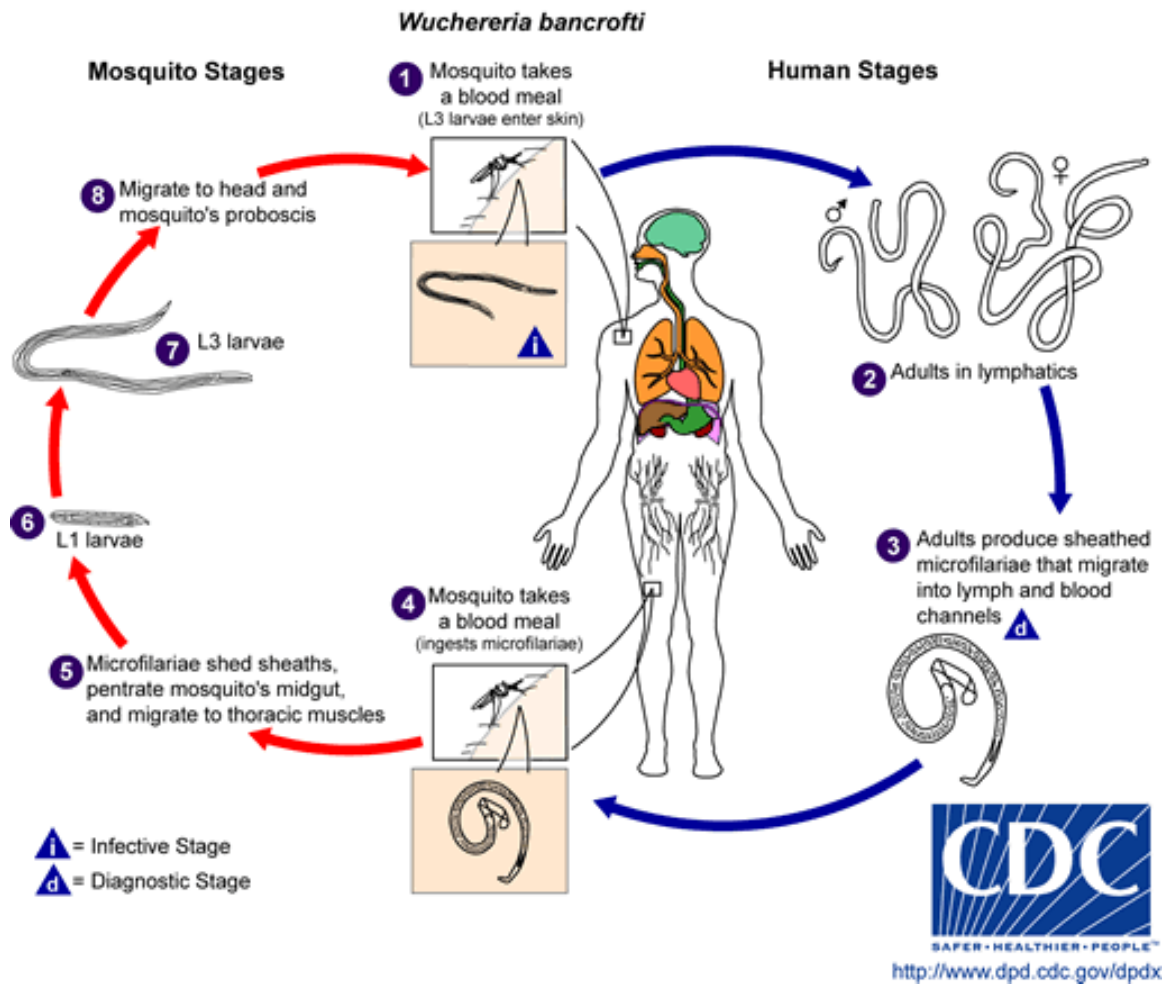
The symptoms associated with LF fall into a broad spectrum and clinical disease is dependent on the host, parasite burden, and parasite strain. Acute infection is often associated with febrile attacks, called “filarial fever.” These fevers begin as a chill, transition to a fever lasting 1-2 days, and leave between days 2-5. Lymphangitis and lymphadenitis may also be present during acute infection. Lymphangitis occurs near a lymph node (LN) and spreads along the lymphatic channel. The lymphatic vessel becomes tender and swollen, with the overlying skin becoming edematous and erythematous. Periodic lymphangitis attacks may occur with rapid, sudden onset, causing the individual severe pain. Lymphadenitis may occur alone or in combination with

Figure 3. Life cycle of filarial worms

W. bancrofti, *B. malayi*, and *B. timori*, the causative agents of LF, have similar life cycles. A mosquito vector transmits infectious L3 larvae to a human host, which then travel to the lymphatics and develop into adults. Adult worms mate and produce MF which circulate in the blood stream. MF are taken up by mosquitoes during subsequent blood meals and develop to the L3 stage in the vector to complete the life cycle. *B. malayi* is capable of infecting mammals other than humans, such as cats, dogs, and monkeys.

Figure obtained from:

http://www.cdc.gov/parasites/lymphaticfilariasis/biology_w_bancrofti.html



lymphangitis. With lymphadenitis, LNs, most commonly the femoral or epitrochlear, become firm, enlarged, and tender (102).

The preferred sites of adult worms are the inguinal and scrotal lymphatics. Inflammation and thickening of the spermatic cord often occurs in response to infection, and hydrocele can develop gradually as interstitial fluid accumulates in the scrotal sack. Rupture of the lymphatics into urinary tract can result in chyluria, and rupture into the scrotal sac can cause chylocele, with individuals often experiencing repeat episodes (2). Interestingly, it is now understood that the majority of filarial fevers are due to bacterial skin and soft tissue infections. When filarial fevers are caused by the worms themselves, retrograde lymphangitis is observed.

Elephantiasis is uncommon, but considered a complication of late infection. Elephantiasis is characterized by enlargement of the limbs, scrotal sac, vulva, or breast tissue. There is loss of skin elasticity and dysfunctional lymphatic flow due to worm obstruction, inflammation, and dilation of lymphatic vessels. This causes fluid to accumulate in the tissues, and most often affects the lower extremities. Elephantiasis and lymphadenitis are exacerbated by repeated secondary bacterial infections. Dysfunctional fluid dynamics of the lymphatic system prevent the host from responding to secondary infections, and further inflammation occurs. Skin thickening on the affected limbs, another hallmark of elephantiasis, tends to occur over time due to poor circulation (2). Because it takes years for pathology to develop, elephantiasis is not often observed in children. Due to the physical deformities that can result from LF, individuals affected by the disease are often ostracized by their communities (246).

While LF can result in devastating morbidity, approximately 60% of infected people are asymptomatic. However, asymptomatic infection does not preclude an individual from disease, as subclinical renal or lymphatic pathology may still exist. Individuals who mount strong immune responses tend to be amicrofilaremic but develop severe lymphatic disease. In contrast, those who experience immune regulation during chronic infection are typically microfilaremic and asymptomatic. Therefore, individuals who do not develop symptoms or an immune response against the infection tend to contribute to disease transmission by providing vectors with an abundant source of microfilaria (169).

Detection

LF infection can be detected by various methods. Microscopic analysis of blood samples collected at night is the standard method for diagnosis of infection. Microscopy also allows for the designation of parasite species based on MF morphology. Provocative administration of the anti-filarial drug diethylcarbamazine (DEC) can induce daytime presence of nocturnal MF if the sample cannot be collected at night (138). A Knott's concentration (109) or nucleopore filtration (2) of the blood sample can be used to increase the sensitivity of microscopic diagnosis when MF numbers are low. LF diagnosis can be made on a clinical basis, however detection of MF in the blood is typically used as confirmation (2).

Serology-based tests are available (e.g. ELISAs) (45), but can be difficult to perform in the field. In 1997, a rapid, card-based diagnostic test (BinaxNOW® Filariasis) was developed to detect circulating adult worm antigen (235). Recently, a new antigen test (Alere Filariasis Test Strip) was developed to increase sensitivity of diagnosis (234).

Unlike MF testing, antigen detection can be performed on blood samples taken from patients at any time of day or night. Finally, ultrasonography can be used to visualize worms in the lymphatic vessels of breast and scrotal tissue (9).

Treatment

Treatment options range based on the clinical disease status of an infected individual. For those suffering from elephantiasis, supportive care can help alleviate some of the symptoms associated with the disease (4). Secondary infections are treated with antibiotics or antifungals. Wearing shoes and regular washing of the affected body parts have been shown to significantly reduce the occurrence of secondary bacterial and fungal infections, as well as lymphangitis attacks (213). Compression bandages and mobilization are prescribed to support fluid drainage from swollen limbs. Surgical excision of large hydroceles may also be performed to help increase patient mobility (2; 4).

DEC, ivermectin, albendazole, and doxycycline are pharmaceutical drugs that are used to treat LF infection. DEC is a microfilaricide but does have some activity against adult worms over time (64). The exact mechanism of action is unknown, however the host immune system is required for MF clearance as *in vitro* DEC treatment fails to kill MF. It is hypothesized that DEC induces the sequestration of MF in organ tissues, ultimately resulting in MF clearance through ADCC (51). Use of DEC is contraindicated for regions that are co-endemic for *Onchocerca* parasites, as treatment has the potential to induce blindness in individuals suffering from onchocerciasis.

Ivermectin is administered as an alternative to DEC in regions with onchocerciasis. Ivermectin targets glutamate-gated chloride channels in the worm,

inducing worm paralysis. Because of its inability to cross the blood-brain barrier, ivermectin has no effect on the host nervous system. Ivermectin is effective at clearing circulating MF and preventing MF release from adult female worms for parasites that cause LF and onchocerciasis (40).

Both DEC and ivermectin are given in conjunction with albendazole for mass drug administration (MDA) efforts (136). Although the effect of albendazole on enhancing DEC and ivermectin clearance of filarial worms is highly debated, albendazole does cure soil-transmitted helminth infections. It is argued that clearing the host of intestinal parasites increases DEC uptake in the intestine, and therefore aids in LF treatment (194).

Doxycycline is a tetracycline antibiotic that has efficacy against bacterial, protozoan, and helminth pathogens. In the setting of helminth infection, doxycycline targets *Wolbachia*, an endosymbiotic bacteria present in filarial worms. Treatment with doxycycline induces worm sterility, and is therefore an effective microfilaricide (86). Adult worm death also occurs, but only when doxycycline is given for 6-8 weeks. Doxycycline also has a positive effect on lymphedema. The mechanism behind this is multifactorial, with the antibacterial and anti-inflammatory properties of doxycycline both likely playing a role in reduced host pathology (137).

Despite the existence of several microfilaricides, to date, no effective short-course macrofilaricides have been discovered. This means that individuals remain infected until adult worms die naturally in 5-7 years, and that pharmaceutical intervention primarily prevents further transmission of the parasite.

Global program to eliminate lymphatic filariasis

Control of LF is problematic because there are no vaccines available for the disease. Natural infection with the parasite does not result in sterilizing immunity (186), providing a difficult framework on which to identify potential candidates for vaccine development. Despite these setbacks, the WHO established the Global Program to Eliminate Lymphatic Filariasis (GPELF) in 2000 with the hopes of achieving elimination of the disease by 2020. There are two arms of the program. The first involves stopping transmission of the parasite to uninfected individuals through MDA, and the second involves caring for individuals who are already infected to reduce suffering associated with the disease (236).

As of 2009, 53 of the 81 countries endemic for LF have begun MDA. MDA consists of yearly treatments with DEC and albendazole, or ivermectin and albendazole, depending on the country's endemicity for onchocerciasis. Pharmaceutical companies have played a large role in the success of the MDA program, with GlaxoSmithKline donating albendazole and Merck & Co. donating ivermectin free of charge. An additional benefit of the GPELF is a reduction in geo-helminth infections due to the use of albendazole during MDA (236).

As substantial progress continues to be made in distributing anthelmintic drugs to endemic regions, the GPELF has begun to focus energy on building platforms for foci mapping and post-MDA surveillance. Both the BinaxNOW® and Alere Filariasis Test Strip are used to monitor the success of MDA treatments in endemic regions, and guidelines state that endemic countries must test for LF for 5 years following MDA treatment to verify elimination of the disease. Egypt is considered the first country to successfully complete the elimination program following 5 years of MDA (150).

The operational and political challenges associated with MDA have highlighted the need for complementary strategies, such as vector control, to eliminate LF (238). Vector-based interventions include residual indoor spraying, insecticide-treated bed nets, and environmental management to limit the availability of water sources for mosquito breeding. Modeling work proposes that vector control has the capacity to significantly reduce the number of years MDA treatment is required to eliminate the disease (21).

Animal model of filarial infection

L. sigmodontis is a rodent model of filariasis used to study host-parasite interactions and the immune responses generated against filarial parasites. *L. sigmodontis* is transmitted by a mite vector and naturally infects the cotton rat *Sigmodon hispidus* (188). *L. sigmodontis* is also able to infect *Meriones unguiculatus* (Mongolian jirds) (Fig. 4A) and immunocompetent BALB/c mice (173). Whereas adult worms develop in the lymphatics in humans, *L. sigmodontis* adults develop in the pleural cavity in rodents. Although clinical disease differs between mice and humans, a similar immune response is seen in both hosts: acute infection is associated with the development of a Th2 response, and chronic infection is associated with the development of immune regulation (139; 220; 221; 223).

Important milestones in parasite development tend to coincide with the development of an immune response against infection (Fig. 4B). L3 larvae injected subcutaneously into susceptible BALB/c mice migrate to the pleural cavity where they become L4 larvae at week 1. At week 4, L4 larvae become adults and mice produce polyclonal IgE. *L. sigmodontis* antigen (LsAg)-specific IgE starts being produced at week 6 when Th2 cytokines IL-4 and IL-5 reach peak levels. Basophil activity rises steadily

throughout acute infection and activation peaks at week 8 when MF are first detected in the blood (223). At week 10, there is a simultaneous decrease in basophil activation and increase in MF production (223). Thus, parasite-induced immune regulation appears to peak at 10 weeks post-infection. Adults begin to die at week 13 and circulating MF densities subsequently decrease. By week 22 the infection is cleared with no adult worms or circulating MF present.

***In vitro* culture of filarial worms**

One of the greatest obstacles hindering the development of helminth-derived therapeutic agents is the identification of antigens that are responsible for eliciting protection against allergic disease. The identification of antigens has primarily been stalled by the limited number of worms available from *in vivo* life cycles. Other setbacks include expenses incurred for antigen production, purification, and the probable need for frequent administration. These difficulties are magnified by our lack of knowledge concerning basic parasite biology and our inability to culture worms *in vitro*. The development of an *in vitro* culture system would provide a cost-effective method for the identification, characterization, and mass production of protective worm antigen. It would also provide consistency among batches, eliminate contamination, and allow for the production of large quantities of worms.

History

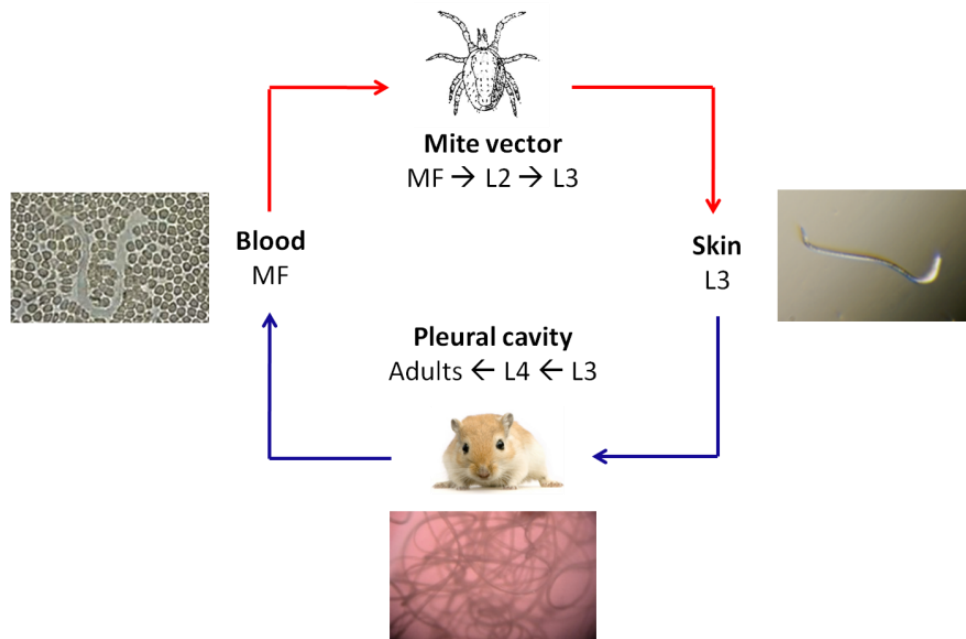
Investigators have been attempting to culture filarial worms *in vitro* since the 1960s, with the majority of studies published in the 60s, 70s and 80s (208). Despite the progress that was made, *in vitro* work halted in the 1990s. Since the start of the GPELF in 2000, there has been a growing interest in the development of additional drugs

Figure 4. *Litomosoides sigmodontis* model of filariasis

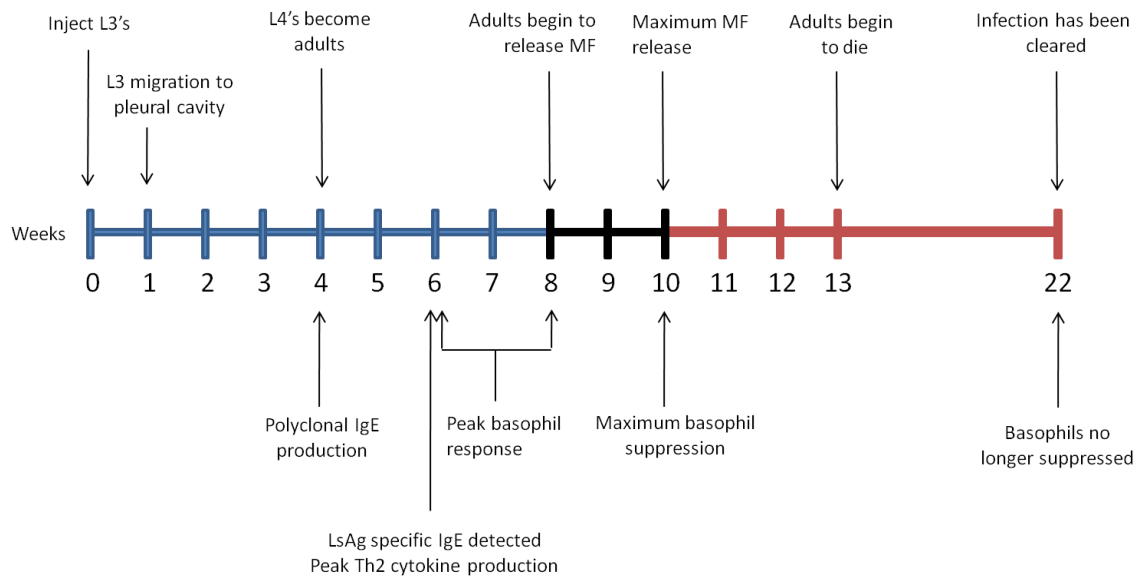
(A) Life cycle of *L. sigmodontis* in *M. unguiculatus*. L3 larvae are transmitted by the bite of a mite vector. Larvae travel to the plural cavity and develop into adults. Adult worms mate and MF circulate in the blood stream. MF are taken up by a mite during a blood meal and develop into L3 larvae in the vector to complete the life cycle.

(B) Murine immune response to *L. sigmodontis* infection. Development of the parasite within the host coincides with key immunologic events. Polyclonal IgE can be detected when L4 larvae become adults at 4 weeks post infection. LsAg-specific IgE can be detected at 6 weeks post infection and peak basophil responses are observed weeks 6-8. At 8 weeks post infection, the time point at which MF are released, the host begins to undergo immune regulation. Immune regulation peaks at 10 weeks post infection and immune cells are hyporesponsive to stimulation. Immune regulation requires active infection, as hyporesponsiveness is gone once the infection has been cleared.

A. Life cycle of *L. sigmodontis* in *M. unguiculatus*



B. Murine immune response to *L. sigmodontis* infection



to combat LF. This recent desire for new macrofilaricides has revitalized efforts to characterize the nutritional requirements of filarial worms. The establishment of an *in vitro* culture system that ensure worm longevity is essential for the high-throughput screening and discovery of novel drug candidates.

Nutritional requirements

Very little is known about the nutritional requirements of filarial worms. Genetic evidence indicates that worms lack pathways for *de novo* synthesis of purines, riboflavin, and heme (44; 70), suggesting they require an external source for these nutrients. Filarial worms also lack pathways for cholesterol synthesis (65), however *W. bancrofti* has been shown to have a putative LDL receptor, thus allowing worms to acquire cholesterol from the host (185). Vitamin A (210) and folic acid (184) are additional nutrients that have specifically been shown to be important for worm survival and development.

MF contain an “inner body” cell group that may function as a food reserve early in development, as it is eventually resorbed (122). MF lack a functional alimentary canal (116) and are thought to absorb nutrients through the cuticle (90), which is permeable to low molecular weight substances. Indeed, experiments have shown that MF are capable of taking up exogenous glucose, nucleic acid precursors, and amino acids (89). L3 larvae have an open alimentary canal but the posterior end of the gut is closed (19).

Mitochondria have been observed in the proximal half of the digestive track, but whether this occurs by chance or the worm is actively digesting host mitochondria is inconclusive (208). L4 and adult larvae have a fully developed functional gut that is capable of taking up exogenous nutrients. Even less is known about the nutritional requirements of filarial worms during development in the vector. Data currently available suggests that worms do

not require host hormones (245) and that the nutritional quality of blood meals affects worm health (184; 209).

Molting

In vitro molting of MF to L2 stage larvae has been achieved with *Dirofilaria immitis*, a parasite that causes dog heartworm, in both cell-free cultures and co-culture with insect cell lines (208). *B. pahangi* and *B. malayi* MF can develop to the L2 stage in presence of mosquito cell lines only if exsheathment is induced prior to culture via proteolytic enzymes or CaCl₂ (46). Ultimately, *in vitro* larval development past the L2 stage has not been observed for any species of filarial parasites (208).

Cultivation of L2 stage larvae can be achieved by isolating whole organs from mosquitoes that have previously fed on microfilaremic blood. *D. immitis* L2 stage larvae can be cultured from the Malphigian tubules (219), and *B. pahangi* can be cultured from thoracic muscles (159). *B. pahangi* L2 larvae have been shown to molt to L3 stage *in vitro* as long as the larvae were of a certain length. This led investigators to speculate that the L2 to L3 molt is dependent on the growth pattern of the larvae (208).

Co-culturing *B. malayi* and *B. pahangi* L3 larvae with a Rhesus monkey kidney cell line (LLCMK₂) resulted in two consecutive molts and the successful development of adult worms *in vitro*. For *B. malayi* the L3 to L4 molt occurs between 14-28 days, and approximately 20% of L4 larvae molt to the adult stage after 44 days in culture. *B. pahangi* larvae suffered from high mortality during the molting process, but this was partially rectified by substituting human serum for 10% FBS (135). The LLCMK₂ culture system has also been used with other species of filarial worms with comparable results (208).

When culturing adult worms, MF release can be used to measure worm fertility. Mating of young adult male and female worms has not yet been achieved *in vitro*, however patent females will continue to release MF *in vitro* after being isolated from the mammalian host. Despite releasing high numbers of MF during the first several days of culture, MF production by adult female worms drops off rapidly and no new embryonic stages are produced *in vitro* (208).

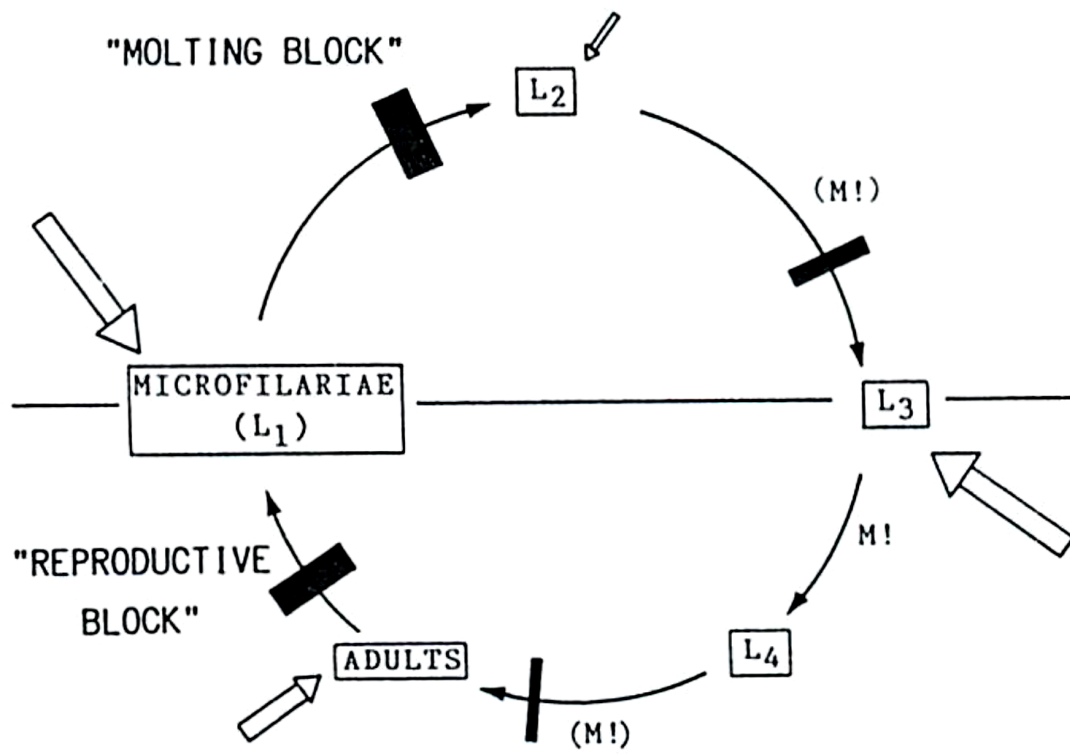
In summary, there are several hurdles to overcome before the development of a complete *in vitro* life cycle is achieved. These hurdles consist of “molting blocks” that prevent further development of a specific larval stage (Fig. 5). The majority of molting success has been observed with the transition of L3 larvae to adult worms. All other stages of the life cycle are impeded by developmental or reproductive blocks (208). Ultimately, the successful development of an *in vitro* life cycle is inhibited by our lack of knowledge of the nutritional and environmental requirements for each molting stage. Once each larval stage can survive *in vitro* for an extended period of time, experiments can focus on the addition of stimuli or supplemental nutrients to trigger molting.

Applications

The development of an *in vitro* culture system that allows filarial worms to mature through all stages of the life cycle outside of an arthropod and mammalian host would have substantial implications for the treatment of both LF and allergic disease. Designated as an NTD, funding sources for filarial research are limited. Thus, replacing definitive and vector hosts would significantly reduce the costs associated with laboratory research. Furthermore, *in vitro* culture systems would remove confounding variables associated with worm development taking place inside other organisms.

Figure 5. Road blocks preventing the development of an *in vitro* life cycle

Filarial worms have successfully molted from L3 stage larvae to adults *in vitro*. Neither mating of adult male and female worms nor oogenesis has been observed *in vitro*, thus, a “reproductive block” exists for adult worms. Additionally, there is a “molting block” that prevents the development of MF to L2 and L3 stage larvae. Cultivation of L2 stage larvae requires the addition of infected mosquito tissue to *in vitro* culture systems. Reprinted with permission from CRC Press, Inc.: copyright 1990 (208).



Compared to bacteria and viruses, little is known about the basic biology of parasitic worms. Understanding the specific nutrients filarial worms require to survive, reproduce, and molt provides a foundation for rational drug development. Specific drugs can be designed to target nutrient uptake, sexual differentiation, reproduction, MF development and release, molting, motility, and migration. Drugs that affect adult worm longevity would be particularly important for the GPELF. Incorporation of macrofilaricidal drugs into the program would drastically shorten the time required to eliminate LF in endemic regions.

The limited number of worms recovered from arthropod and mammalian hosts restricts the number and type of experiments that can be performed in the laboratory. An *in vitro* culture system would allow for the mass production of specific stages of the parasite. At this time, whole worm antigen (WA) and excretory-secretory product (ESP) are scarce resources. This is problematic as WA and ESP have been shown to be immunomodulatory and can protect animals from allergic disease (81; 85).

Characterization of the specific antigens responsible for protection will be stalled until there is a method to collect high volumes of worm products. Because infection with filarial worms can induce pathology, the use of worm antigen as a therapeutic for allergic disease is highly preferred.

STATEMENT OF PROBLEM

Allergic hypersensitivity reactions cause significant disease burden in developed nations, including morbidity, mortality, strain on health care systems, and economic loss. Studies have shown an inverse relationship between helminth infections and allergic disease. However, there are major obstacles that prevent helminths from being used as a therapeutic agent for hypersensitivity reactions. 1) There is conflicting evidence regarding the therapeutic potential of helminth infections for pre-existing allergic disease. The majority of animal studies completed to date demonstrate that infection can prevent the development of allergies, but few have investigated whether infection can ameliorate allergic symptoms associated with established allergy. 2) Studies have primarily focused on helminth protection against type I and type IV hypersensitivities. Protection against type III hypersensitivity has yet to be evaluated. 3) Filarial worms can cause the host significant pathology, which makes the use of protective worm antigens an attractive alternative to live infection. Due to the complex life cycle of parasitic helminths, which relies on both mammalian and vector hosts, we are limited in our ability to produce and characterize protective worm antigens. This highlights the need for an *in vitro* life cycle so that specific larval stages can be expanded and mass-produced with high consistency

My work seeks to address each of these major obstacles, first by testing whether chronic helminth infection alters the immunological markers of type I hypersensitivity and whether it can be used as a therapeutic for clinical allergic disease. My research also explores the effects helminth infection has on type III hypersensitivity, as protection against this type of allergy has not yet been assessed. Finally, a concurrent goal of my work is to determine culture conditions that support the *in vitro* survival of filarial

worms. Prolonged *in vitro* survival is the first step toward the development of a self-sustaining *in vitro* life cycle.

SPECIFIC AIMS

Specific Aim 1: Determine whether chronic helminth infection can be used as a therapeutic for pre-existing allergic disease.

Subaim 1: Determine whether chronic *L. sigmodontis* infection protects against pre-existing type I hypersensitivity

- Hypothesis: Immunological markers of allergy, including basophil activation, mast cell degranulation, and levels of allergen-specific IgE will be suppressed in the setting of chronic helminth infection.
- Hypothesis: Infection will protect against early and late phase clinical allergic responses due to mast cell and basophil suppression.

Subaim 2: Determine whether chronic *L. sigmodontis* infection protects against type III hypersensitivity.

- Hypothesis: Chronic infection will protect against ear swelling during a cutaneous Arthus reaction.

Subaim 3: Determine the mechanism(s) by which infection protects against type III hypersensitivity.

- Hypothesis: Infection reduces ear swelling by preventing cellular infiltration following allergen challenge.
- Hypothesis: Cellular infiltration will be impaired due to decreased chemokine production.

Specific Aim 2: Develop culture methodology that supports the *in vitro* survival of filarial worms.

Subaim 1: Determine whether co-culturing MF with cell lines enhances *in vitro* survival

- Hypothesis: Since MF typically survive for months in the mammalian host, co-culturing MF with endothelial cells will enhance *in vitro* survival.

Subaim 2: Characterize the factors that MF require for survival

- Hypothesis: MF will likely require multiple factors produced by endothelial cells, including both protein and lipid components.

Subaim 3: Determine if the culture conditions that prolong MF survival also prolong adult worm survival

- Hypothesis: Adult filarial worms will require similar nutrients as MF for *in vitro* survival.

CHAPTER 2: Materials and methods

HELMINTH INFECTION AND HOST PROTECTION FROM ALLERGY

Animals

4-6 week old female BALB/c mice (National Cancer Institute Mouse Repository, Frederick, MD), IgE-deficient mice that lack the IgE heavy chain (The Jackson Laboratory, Bar Harbor, ME), C57BL/6 mice (The Jackson Laboratory), W^{sh} mice (The Jackson Laboratory), and J_H^{-/-} mice (Taconic, Hudson, NY) were housed at the Uniformed Services University Center for Laboratory Animal Medicine. All experiments were performed under protocols approved by the Uniformed Services University Institutional Animal Care and Use Committee.

Ovalbumin sensitization

Mice were sensitized as previously described (54). In brief, mice received i.p. injections of 50 µg of ovalbumin (Sigma-Aldrich) adsorbed to 2 mg aluminum hydroxide (Pierce) in PBS on days 0, 7, and 14. Mock sensitization groups received i.p. injections of 2 mg aluminum hydroxide in PBS. Mice were given a 2-6 week rest period before infection or ovalbumin (OVA) challenge.

***Litomosoides sigmodontis* infection**

Infectious L3-stage larvae were isolated from the pleural cavity of infected jirds (*Meriones unguiculatus*, obtained from TRS Laboratory, Athens, GA) as previously described (Hubner 2009). Mice were infected by subcutaneous injection of 40 L3 larvae in RPMI-1640. Mock treatment groups were given a subcutaneous injection of RPMI-

1640 (Mediatech, Inc.). Worm counts, OVA challenge and immunological assays were performed 10 weeks post-infection.

Intradermal OVA challenge

The local anaphylaxis assay was performed as previously described (54). Mice were given an intradermal injection of 20 µg OVA in 10 µl of PBS in one ear and 10 µl of vehicle alone (PBS) in the other ear. Three minutes after challenge, 200 µl of 0.5% Evans Blue dye (Sigma-Aldrich) was injected into the tail vein. Ten minutes later, animals were euthanized with CO₂ and ears were removed and placed in formamide (Sigma-Aldrich) overnight at 63°C. Extracted dye was measured at an absorbance of 620 nm. The O.D. of the vehicle challenged ear was then subtracted from the O.D. of the OVA challenge ear for each animal.

For the ear thickness assay, a micrometer was used to measure baseline ear thickness prior to i.d. injection of 20 µg OVA or vehicle. Ear thickness was then measured at 1, 2, 12, 24, 48, 72, and 96 hours post challenge.

Basophil, CD4⁺ T cell, and platelet depletions

For basophil depletion, mice received an i.p. injection of 50 µg of anti-CD200R3 clone Ba103 (Hycult Biotech) or rat IgG2b isotype control clone A95-1 (BD Biosciences) 24 hours before challenge. For CD4⁺ T cell depletion, mice received an i.p. injection of 500 µg of anti-CD4 clone GK1.5 (BioXcell) or rat IgG2b isotype control 24 hours before challenge. Depletion of basophils and CD4⁺ T cells was confirmed via flow cytometry 4 days after challenge. For platelet depletion, mice received an i.p. injection of 4 µg of anti-CD41 clone MWReg30 (provided by John Semple M.D., University of Toronto) or rat IgG1 isotype control clone R3-34 (BD Biosciences) 24 hours before challenge. Platelet

depletion was confirmed by complete blood count (CBC) 24 hours after administration of the antibody.

Histology

Ten weeks post-infection, animals were challenged with PBS in one ear and 20 µg OVA in the other. Three and twenty-four hours after challenge, animals were euthanized and ears were fixed in 10% formalin. H&E and toluidine blue staining was performed by Histoserv (Rockville, MD). Slides were digitized with a 20X objective on a 2.0-RS NanoZoomer (Hamamatsu) using NDP.scan software version 2. Digitized slides were analyzed with NDP.view software version 1 and the number of cells staining positive for toluidine blue were counted. A blinded pathologist (B. K. Mitre) then scored each of four parameters (hemorrhage, edema, necrosis and cellularity) on the basis of severity (0-absent, 1-mild, 2-moderate, or 3-severe) and focality (0-absent, 1-focal, 2-intermediate, and 3-diffuse) for a total maximum inflammation score of 24.

Immunohistochemistry

Ten weeks post-infection, animals were challenged with PBS in one ear and 20 µg OVA in the other. Three hours after challenge, animals were euthanized and ears were frozen with liquid nitrogen. Immunohistochemistry was performed by Histoserv (Rockville, MD). Ears were stained with anti-IgG FITC (Sigma) or DAPI, anti-C3 FITC (Immunology Consultants Laboratory, Inc.), and anti-IgG Texas Red (Life Technologies). Confocal images of the entire ear were obtained with a 10X objective using a Zeiss 710 microscope and Zen software. Prior to analysis, TIFF files were exported to Adobe Photoshop and non-specific staining by anti-C3 FITC along the outline of the ear was masked to ensure proper immune complex identification (Fig.

12A). Analysis of immune complexes was performed using the Puncta Analyzer plugin v2.0 (<https://github.com/physion/puncta-analyzer>) in ImageJ (imagej.nih.gov/ij/).

ELISAs and ELISPOT assays

ELISAs were performed on plasma samples from 10-week infected mice. Total IgE (eBioscience), total IgG (eBioscience), total IgG2a (Kamiya Biomedical Company), OVA-specific IgE (MD Bioproducts), OVA-specific IgG1 (Caymen Chemical), and OVA-specific IgG2a (Alpha Diagnostic) ELISAs were performed according to manufacturer instructions. Absorbance was detected with a Victor³ V microplate reader (PerkinElmer).

For ELISPOT assays, single cell suspensions of splenocytes were prepared for each animal at 10 weeks post-infection. Spleens were removed, homogenized through a 0.7 μ m cell strainer (BD), and RBCs were lysed with ACK lysing buffer (Quality Biological, Inc.). Cells were frozen in RPMI-1640 containing 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, 25 mM Hepes buffer, 30% fetal calf serum and 10% DMSO. Total and OVA-specific IgG2a ELISPOTs (U-CyTech Biosciences) were performed according to manufacturer instructions. Spots were counted manually with a dissecting microscope.

CBCs

Ten weeks post infection, whole blood was collected into EDTA tubes (BD) and complete blood counts (CBCs) were obtained by BioReliance (Rockville, MD).

Obtaining cells from ear tissue

Tissue processing methodology was adapted from Shannon et al. 2013 (201). Both ears were challenged by i.d. injection of 20 µg OVA. 24 hours later, mice were euthanized and a terminal bleed was performed. Then, animals were perfused with 5 ml PBS by intracardiac injection, after which ears were removed and rinsed with 70% ethanol. The dorsal and ventral dermal layers of both ears were then separated and placed in a 35 x 10 mm non-tissue culture treated petri dish (BD) containing 3 ml digestion buffer (RPMI-1640, 25 mM HEPES [Mediatech, Inc.], 1.5 g/L NaHCO₃, 100 U/ml DNase I [Roche], and 170 µg/ml Liberase TM [Roche]). Ear tissue was incubated at 37°C for 30 minutes and homogenized through a 0.7 µm cell strainer to create a single cell suspension. 4 ml of PBS was used to rinse the strainer and additional 3 ml of PBS was added to bring the final sample volume to 10 ml. Cell counts were obtained with a Countess automated cell counter (Life Technologies) using trypan blue exclusion.

Basophil activation assay

Ten weeks post-infection, whole blood was collected into heparinized tubes (BD). Samples were centrifuged at 600 x g for 10 minutes, plasma was removed, and remaining cells were washed with RPMI-1640. Washed cells from two animals were pooled for each sample. Samples were stimulated with RPMI-1640 and 40, 10, 2.5 and 0.625 µg/ml ovalbumin for 1 hour at 37°C and 5% CO₂. GolgiStop (BD Biosciences) was added and cells were incubated for an additional 2 hours at 37°C. Cells were washed twice with PBS, lysed, and fixed with a whole blood lysing kit (Beckman Coulter). Basophil activation was assessed by flow cytometry.

Flow cytometry

To measure basophil activation, fixed cells were blocked with 1% PBS/BSA for 1 hour at 4°C and then incubated with Perm/Wash buffer (BD Biosciences) for 15 minutes at 4°C. Cells were stained with anti-IgE FITC clone R35-72 (BD Biosciences), anti-CD4 PerCP clone RM4-5 (BD Biosciences), anti-B220 PerCP clone RA3-6B2 (BD Biosciences), and anti-IL-4 APC clone 11B11 (BD Biosciences). Basophils were gated as CD4⁻B220⁻IgE⁺, and activated basophils were gated as IL-4⁺. 1×10^5 events were analyzed per sample.

To determine cell types recruited to the ear following OVA challenge, single cell suspensions of ear tissue were centrifuged at 290 x g for 5 minutes and resuspended in 500 µl of 1% PBS/BSA for 30 min at 4°C. Cells were stained with anti-Ly6G APC-eFluor780 clone RB6-8C5 (eBioscience), anti-F4/80 Pacific Blue clone BM8 (eBioscience), anti-CD19 PE-Cy5 clone eBio1D3 (eBioscience), anti-CD11c BV421 clone N418 (Biolegend), anti-SiglecF PE-CF594 clone E50-2440 (BD Bioscience), and anti-CD45 APC-Cy7 clone 30-F11 (Biolegend), and fixed in 4% paraformaldehyde. Eosinophils were gated as CD11c⁻CD45⁺SiglecF⁺, dendritic cells were gated as CD11c⁺, B cells were gated as CD19⁺, macrophages were gated as F4/80⁺, and neutrophils were gated as F4/80⁻Ly6G⁺. 1.5×10^4 events were analyzed for mock treated animals, 3×10^4 events for infected animals, 4×10^4 events for sensitized animals, and 4×10^4 events for sensitized + infected animals.

To measure FcγR expression, aliquots of previously prepared frozen splenocytes were thawed and resuspended in 500 µl of 1% PBS/BSA for 30 min at 4°C. Samples were stained with the aforementioned antibodies to determine cell types recruited to the ear. Additionally, samples were also stained with anti-CD64 PE clone X54-5/7.1

(Biolegend), anti-CD32 (Biorbyt) conjugated to FITC (EasyLink FITC conjugation kit, Abcam), and anti-CD16 clone EPR4333 (Abcam) conjugated to APC (Lightning-Link APC antibody labeling kit, Novus Biologicals). Cell types were gated as previously described. Each cell type was then assessed for FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) positivity. 1×10^6 events were analyzed per sample.

For all flow cytometric experiments, antibodies were individually titrated using splenocytes from naïve BALB/c mice and OneComp eBeads (eBioscience) were used to calculate compensation for each flow cytometry run. Gates were established using the fluorescence-minus-one technique. Flow cytometric data was collected with a BD LSR-II flow cytometer (BD Biosciences) and analyzed with FlowJo software version 7 (TreeStar).

Chemokine production

Ten weeks post-infection, both ears were challenged with 20 µg OVA. 6 hours later, mice were euthanized and ears were removed and cut into pieces approximately 1.5 cm in size. Ears were pooled for each mouse and added to Lysing Matrix D FastPrep tubes. One Complete Mini protease inhibitor cocktail tablet (Roche) was dissolved in 10 ml PBS, and 500 µl was added to each tube. Samples were homogenized with a FastPrep-24 instrument (MP Biomedicals) set at speed 5 for 20 seconds. Homogenization was repeated 3 times with a 30-second break between runs. Tubes were then centrifuged at $16,100 \times g$ for 10 minutes, and supernatants were collected and stored at -20°C . Samples were tested for the presence of chemokines with a Proteome Profiler Mouse Chemokine Array Kit (R&D Systems, Inc.) according to manufacturer instructions. Membranes were developed for 10 minutes, and digitized for analysis on ImageJ. Densitometry was

performed using particle analysis, with measurements for each sample spot averaged and then normalized to membrane reference spots.

***In vitro* immune complex stimulation**

Peritoneal macrophages were isolated by performing a peritoneal lavage with 10 ml HBSS. Cells were pelleted and resuspended in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 20 mM Hepes buffer. 2.5×10^5 peritoneal cells in 500 µl RPMI were aliquoted to 48 well plates. Cells were incubated at 37°C for 2 hours to allow macrophages to adhere. Non-adherent cells were removed by washing 3X with 1 ml PBS. 500 µl of fresh RPMI media was added to each well.

A single cell suspension of splenocytes was prepared by homogenizing spleens through a 0.7 µm cell strainer and removing contaminating RBCs by hypotonic lysis. 2.5×10^5 splenocytes in 500 µl RPMI were aliquoted to 48 well plates. To obtain non-adherent splenocytes, cells were incubated at 37°C for 2 hours, after which supernatants were transferred to a new well.

Remaining splenocytes were used to generate an eosinophil-enriched cell suspension via negative selection. Splenocytes were incubated with CD45R (B220) and CD90 (Thy1.2) magnetic beads (MACS, Miltenyi Biotec) and applied to LD columns as per manufacturer instructions. 2×10^5 recovered cells in 500 µl RPMI were aliquoted to 48 well plates. A cytopsin and DiffQuick stain was performed to assess the composition of recovered cells. Preparations typically resulted in 33% eosinophils, 57% PMNs, and 10% monocytes.

Rabbit anti-ovalbumin antibody (Polysciences, Inc.) was resuspended in ultra-pure H₂O and passed through a Mustang E endotoxin removal filter (Pall Corporation). OVA was resuspended in RPMI-1640 and also passed through a Mustang E filter. Immune complexes were generated by incubating 1 µg OVA with 100 µg rabbit anti-ovalbumin antibody, with the total volume brought to 25 µl with RPMI-1640. OVA and anti-ovalbumin antibody were incubated 37°C for 30 minutes, after which 25 µl of immune complexes were added per well of 2 x 10⁵ cells. As a control, additional cells were stimulated with 25 µl of 1 µg of OVA in RPMI-1640. Cells were stimulated with OVA or immune complexes at 37°C for 6 hours, after which plates were centrifuged at 400 x g for 10 minutes. Supernatants were collected and assayed with mouse KC and MIP-2 ELISAs (Ray Biotech) as per manufacturer instructions.

Murine model of systemic lupus erythematosus (SLE)

Mice were given a 0.5 ml i.p. injection of pristane (Sigma-Aldrich). Fourteen weeks after injection, the mice were given s.c. injection of 40 L3-stage *L. sigmodontis* larvae or RPMI-1640. Mice were bled by tail vein prior to pristane treatment, and once per month for 6 months. Concurrent with tail vein bleeds, proteinuria was monitored with Albustix reagent strips (Siemens). Ten weeks post infection (24 weeks after pristane treatment) animals were euthanized and kidneys were harvested for H&E staining (performed by Histoserv). A blinded pathologist (M. A. Smith) scored 50 glomeruli per mouse on a scale of 1-5 (0-normal, 1-mesangial expansion, 2-endocapillary proliferation, 3-capillaritis or necrotic changes, or 5-crescents). Cells were counted in glomeruli that exhibited mesangial expansion. Autoantibody production was determined by anti-dsDNA

and anti-nRNP IgG ELISAs (Alpha Diagnostic), performed according to manufacturer instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 6 (GraphPad Software, Inc.). Sensitized and Sensitized + Infected groups were analyzed using the Mann Whitney test. p values < 0.05 was considered significant. Unless otherwise noted, data is representative of two individual experiments with 4-5 animals per group.

***IN VITRO* CULTURE OF FILARIAL WORMS**

***Litomosoides sigmodontis* microfilariae isolation**

Microfilariae (MF) were isolated from infected jirds (*Meriones unguiculatus*) obtained from TRS Laboratory (Athens, GA). A terminal bleed was performed and blood was collected into a heparinized microcentrifuge tube (BD) and added to 2 ml of RPMI-1640 (Mediatech, Inc.). MF were then isolated from the blood via percoll gradient centrifugation as previously described (29). Isolated MF were resuspended in 1-2 ml RPMI and counted on a hemacytometer. For all experiments, MF were cultured at a concentration of 2×10^4 MF/ml.

Cell lines and culture conditions

A mouse endothelial cell line (EOMA) (CRL-2586), mosquito cell line (C6/36) (CRL-1660), myeloma cell line (Sp2/0-Ag14, CRL-1581) and rat basophil-like cell line (RBL-2H3) (CRL-2256) were obtained from the American Type Culture Collection (ATCC). EOMA and myeloma cells were maintained in Dulbecco's Modified Eagles

Medium (DMEM) and RBL-2H3 cells were maintained in Iscove's Modification of DMEM (IMDM) at 37°C and 5% CO₂. C6/36 cells were maintained in Eagles Minimum Essential Medium (EMEM) and cultured at 28°C and 5% CO₂. All cell culture media were supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin unless otherwise noted. Cell culture reagents were purchased from MediaTech, Inc.

Transwell plates

For transwell plate culture, EOMA cells in 2 ml of supplemented DMEM was added to the bottom reservoir of 0.4 µm transwell plates (Corning, Inc.). MF in 2 ml of supplemented DMEM was then added to the top reservoir.

Conditioned media

Cells were passaged into 182 cm² vented tissue culture flasks (Cell Treat). Twenty-four hours after passage, media was removed and 50 ml fresh media was added. Three days later, the conditioned media (CM) was harvested and centrifuged at 400 x g for 10 minutes. The supernatant was collected, pooled, and sterile filtered. CM was flash frozen in a dry ice and ethanol slurry and aliquots were stored at -80°C for future use. For some experiments, EOMA CM was prepared in the absence of FBS by growing cells for three days in DMEM supplemented with 2 mM L-glutamine and 100 U/ml penicillin/streptomycin.

Heat treatment

Samples were placed in a dry heat bath set at 56°C or 100°C. Samples were incubated for 1 or 4 hours, cooled to room temperature, and then supplemented with 100

U/ml of penicillin/streptomycin. For some experiments, 10% FBS was added after heat treatment.

Lipid depletion

Lipid removal agent (LRA) (Advanced Minerals) was added to 10 ml EOMA CM and DMEM for a final concentration of 40 mg/ml. Samples were agitated at room temperature for 22 hours and then centrifuged at 4,696 x g (5,000 RPM) for 10 minutes. Supernatants were removed and centrifuged again at 4,696 x g (5,000 RPM) for 10 minutes. Supernatants were then passed through a 0.22um filter, flash frozen, and stored at -80°C.

Dialysis

Five ml of EOMA CM was added to 1000 kDa, 20 kDa, 0.5-1 kDa, and 0.1-0.5 kDa Float-A-Lyzer G2 dialysis devices (Spectrum Laboratories, Inc.) pre-conditioned as per manufacturer's protocol. Dialysis devices were placed in 600 ml dialysis buffer (DMEM supplemented with 10% FBS, 2mM L-glutamine, and 100 U/ml penicillin/streptomycin) with a stir bar on a magnetic plate at room temperature. Buffer was exchanged at 2, 4, and 6 hours. Eighteen hours after the last buffer exchange, CM was harvested, flash frozen, and stored at -80°C.

2D DIGE protein expression profiling

Aliquots of frozen EOMA CM and RBL CM were sent to Applied Biomics (Hayward, CA) for 2D DIGE protein expression profiling. The spots that showed greater than 1.5-fold upregulation in EOMA CM compared to RBL-2H3 CM were identified by

mass spectrometry. A cluster analysis was then performed on the identified proteins to determine which functional groups (GO terms) were enriched in EOMA CM.

Culture supplements

In place of FBS, 10% Cell-Ess® (Essential Pharma) or 1X fatty acid supplement (Sigma) was added to DMEM with 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. To supply exogenous purines, EmbryoMax Nucleosides (Millipore) was added at a 1X concentration to DMEM with 10% FBS, L-glutamine and penicillin/streptomycin. Finally, 10 µg/ml cholesterol (Sigma), 20 µg/ml yeast-derived recombinant mouse C3 (MyBiosource), 20 µg/ml yeast-derived recombinant apolipoprotein E (MyBiosource), and 20 µg/ml native albumin (MyBiosource) were added individually to DMEM with 10% FBS, L-glutamine, and penicillin/streptomycin.

Adult worm culture

Adult *L. sigmodontis* worms were isolated from the plural cavity and adult *Brugia malayi* worms were isolated from the peritoneal cavity of infected jirds obtained from TRS Laboratory. For transwell plate experiments, EOMA cells in 2 ml of supplemented DMEM was added to the bottom reservoir of 0.4 µm transwell plates, and 1 adult female in 2 ml of supplemented DMEM was added to the top reservoir. For conditioned media experiments, 1 adult female worm was cultured in 1 ml CM and media was exchanged every other day.

CHAPTER 3: Ten week infection with a tissue-invasive helminth protects against immune complex-mediated inflammation but not clinical type I hypersensitivity in previously sensitized mice

INTRODUCTION

Despite numerous epidemiologic and animal studies suggesting helminth infections are protective against allergy, the two prospective human clinical trials that have tested the efficacy of infection as a therapeutic have failed to show clinical benefit (15; 57). Lack of protection may be due to a variety of factors, including the possibility that helminth infections are better at preventing allergy than treating it. Interestingly, while over 30 animal studies have demonstrated that helminth infection established prior to sensitization protects against the development of allergy, very few have investigated the use of helminths as therapeutics for pre-existing allergic disease (reviewed by Evans et al. 2014) (55).

In this study, we sought to determine whether *L. sigmodontis*, a tissue-invasive filarial nematode that establishes chronic infection in immunocompetent BALB/c mice (87), protects against local hypersensitivity responses after sensitization has taken place. Similar to other helminths, *L. sigmodontis* induces systemic immunomodulation (175; 220; 221), and a previous study demonstrated that *L. sigmodontis* can inhibit the development of allergic disease when infection is established prior to allergic sensitization (47). As we recently demonstrated that chronic *L. sigmodontis* infection suppresses the IgE-mediated activation of basophils (114), we hypothesized that infection may also protect against allergic disease in previously sensitized mice.

Our results demonstrate that while 10 weeks of *L. sigmodontis* infection suppresses numerous immunologic markers of type I hypersensitivity, including allergen-specific IgE as well as basophil and mast cell degranulation in response to allergen, it does not confer clinical benefit as measured by increases in local vascular permeability. Interestingly, though, we did find that infection protects the host from ear swelling due to type III (immune complex mediated) hypersensitivity. This protection is associated with reduced neutrophil-specific chemokine production, fewer neutrophils trafficking to the site of immune complex deposition, reduced chemokine production by eosinophils after immune complex stimulation, and decreased Fc gamma receptor I (FcγRI) expression on eosinophils. Additionally, using a murine model of systemic lupus erythematosus (SLE), another type III hypersensitivity disease, we show that infection reduces levels of anti-dsDNA antibodies and mesangial expansion.

RESULTS

Chronic *Litomosoides sigmodontis* infection does not protect against cutaneous type I hypersensitivity

To determine whether chronic helminth infection protects against type I, IgE-mediated hypersensitivity, mice were sensitized to OVA for 3 weeks, given a rest period, infected with the rodent filarial parasite *L. sigmodontis* for 10 weeks, and then assessed for local anaphylaxis after intradermal injection of OVA (Fig. 6A). Adult *L. sigmodontis* worms reside in the pleural cavity and microfilariae circulate in the blood. In all experiments, sensitization did not affect worm burdens, which were assessed at the study endpoint (Fig. 7).

OVA-sensitized animals had elevated total and OVA-specific IgE levels compared to Mock animals (Fig. 6B, C), confirming that OVA sensitization successfully

elicited a type 2 immune response. As expected, all infected animals had high levels of total IgE (Fig. 6B). Despite having higher levels of total IgE than Sensitized mice, Sensitized + Infected animals had significantly lower levels of OVA-specific IgE compared to Sensitized animals (Fig. 6C).

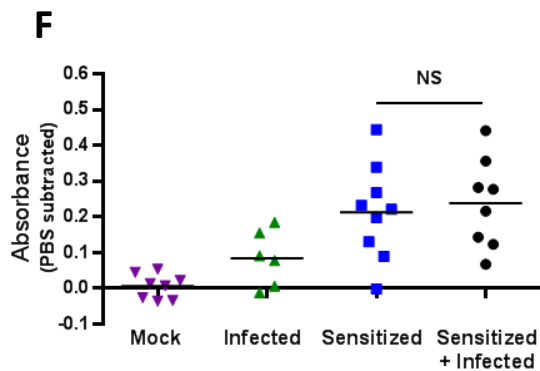
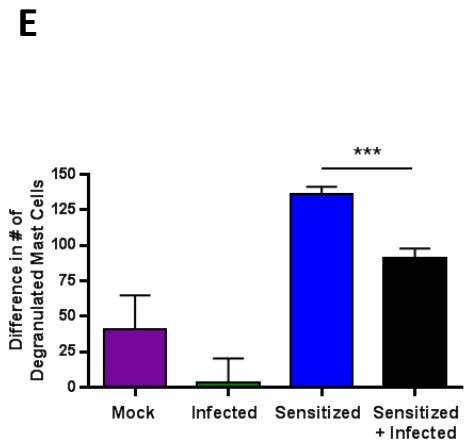
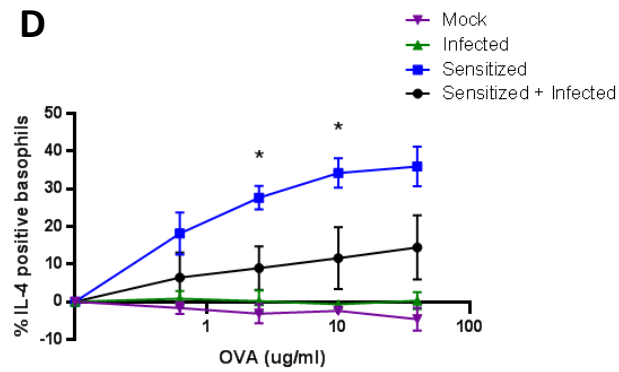
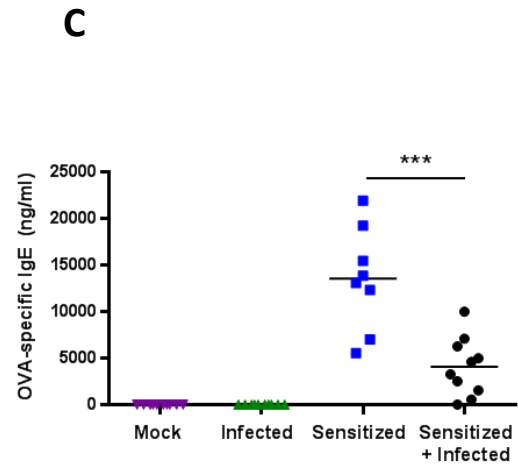
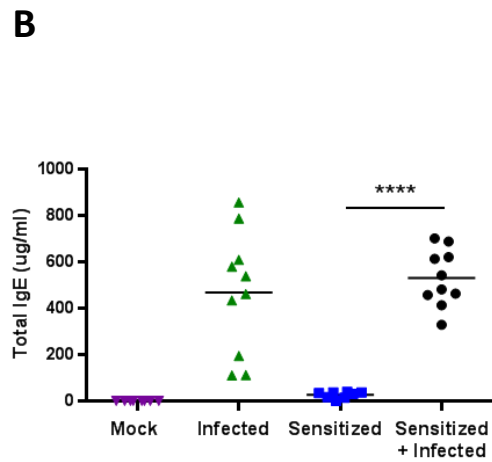
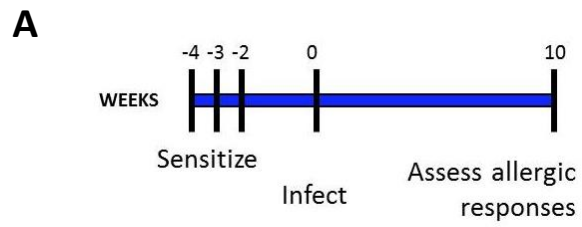
Basophils and mast cells are two major allergy effector cells that release histamine and other pro-inflammatory mediators in response to IgE signaling. To determine if basophils were suppressed in response to allergen, whole blood was stimulated with increasing concentrations of OVA and basophil activation was assessed by measurement of IL-4 production by intracellular flow cytometry. Basophils, identified as CD4⁺B220⁺IgE⁺ cells, had reduced IL-4 production in Sensitized + Infected animals compared to Sensitized animals (Fig. 6D).

To evaluate whether mast cell function was reduced in the setting of chronic *L. sigmodontis* infection, mast cell degranulation was measured by enumerating total and degranulated mast cells in ear tissue 24 hours after intradermal challenge with OVA or PBS. The number of mast cells in the OVA-challenged ear was then subtracted from the PBS-challenged ear. As seen in Figure 6E, chronically infected animals had significantly fewer degranulated mast cells after OVA challenge than Sensitized animals.

Given that Sensitized + Infected animals had lower levels of OVA-specific IgE, decreased IL-4 production by basophils, and reduced mast cell degranulation following OVA challenge, we next tested whether infection protects against clinical allergic responses by using a local anaphylaxis assay (LAA). The LAA monitors changes in vascular permeability by quantifying dye extravasation in the tissue following allergen challenge (54). Sensitized and Sensitized + Infected animals did not have any difference

Figure 6. Protection against type I hypersensitivity

Chronic *L. sigmodontis* infection did not protect against clinical type I hypersensitivity. **(A)** Experimental design for the use of *L. sigmodontis* as a therapeutic agent for pre-existing allergic disease. Levels of circulating total IgE **(B)** and OVA-specific IgE **(C)** were assessed by ELISA at 10 weeks post-infection. **(D)** Flow cytometric analysis of basophil activation in response to increasing concentrations of OVA stimulation. Basophils were gated as CD4⁻B220⁻IgE⁺, and activation was determined by intracellular IL-4 staining using fluorescence minus one controls. Values plotted represent % IL-4⁺ basophils after OVA stimulation minus % IL-4⁺ basophils after culture in media alone. Data is representative of two independent experiments with two animals pooled per sample and media levels subtracted. Sensitized and Sensitized + Infected groups were compared at each concentration by the Mann Whitney test. **(E)** Differences in mast cell degranulation between PBS and OVA challenged ears. Degranulated and non-degranulated mast cells were enumerated by toluidine blue stain of ear tissue 24 hours after challenge. **(F)** Local anaphylaxis assay (LAA) quantification of cutaneous type I hypersensitivity reactions in response to OVA challenge, with PBS values subtracted. Error bars represent \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



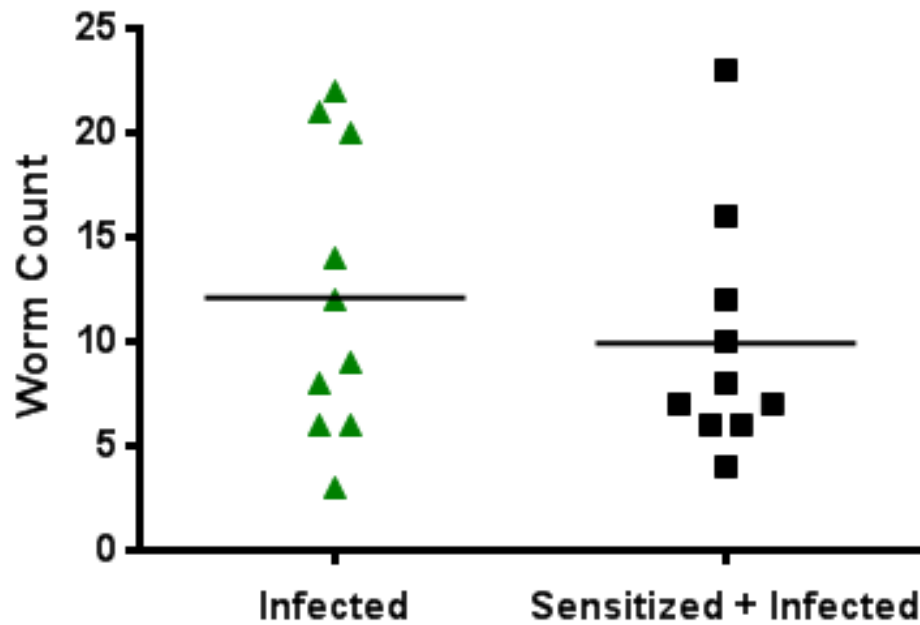


Figure 7. Worm burden at 10 weeks post infection

L. sigmodontis adult worms were recovered from the peritoneal and pleural cavity of infected mice at 10 weeks post infection. There was no significant difference in worm recovery between groups.

in dye extravasation (Fig. 6F), indicating that 10 weeks of infection with *L. sigmodontis* does not protect against the symptoms of type I hypersensitivity in previously sensitized mice. These results suggest that although *L. sigmodontis* suppresses several immunological drivers of type I hypersensitivity (allergen-specific IgE, basophil activation, and mast cell degranulation), the extent to which it does so is not sufficient to appreciably alter clinical responses that occur after local allergen challenge.

Chronic *L. sigmodontis* infection protects against late phase ear swelling due to type III hypersensitivity

We next assessed the effect chronic helminth infection had on late phase inflammation after intradermal allergen exposure. Animals were challenged with 20 µg OVA in the right ear and PBS in the left ear, and ear thickness was serially measured over a period of four days using a micrometer.

Unsensitized animals (Infected and Mock groups) did not respond to OVA challenge and had negligible ear swelling throughout the time course (Fig. 8A). During the early time points (1 and 2 hours after challenge) Sensitized and Sensitized + Infected animals exhibited the same degree of swelling, confirming results from the LAA that infection does not suppress immediate allergic responses. However, at all of the late phase time points (12-96 hours post-challenge) Sensitized + Infected animals had significantly less ear swelling than Sensitized animals (Fig. 8A).

To determine whether late phase ear swelling was due to type I hypersensitivity, we performed the ear thickness assay on mast cell-deficient, IgE deficient, and C57BL/6 control mice. When OVA-sensitized mice from each strain were challenged with OVA, they exhibited increases in ear thickness comparable to sensitized wild-type mice (Fig.

8B). These data indicate that neither IgE nor mast cells are required for late phase swelling.

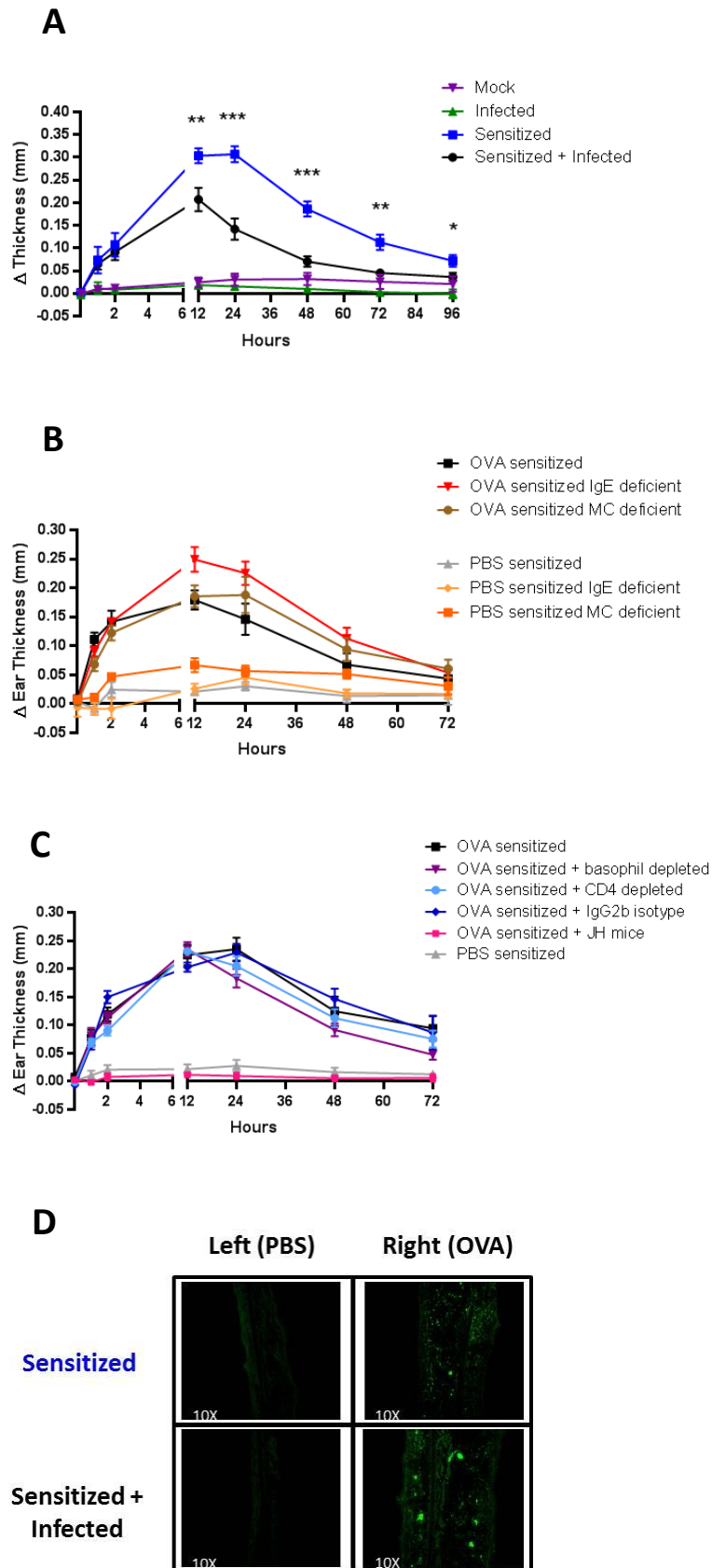
Because previous studies have implicated basophils as important mediators of late phase swelling in type I hypersensitivity responses (152), we next evaluated whether basophils were contributing to the late phase swelling observed in our allergy model. To deplete basophils, BALB/c mice were given 50 µg anti-CD200R3 24 hours prior to OVA challenge. Flow cytometry demonstrated that this approach resulted in >85% basophil depletion for the duration of the monitoring period (Fig. 9). As seen in Figure 8C, basophil depletion did not reduce late phase ear swelling in response to intradermal OVA challenge, indicating that basophils were not important for swelling in our allergy model. Similar results were found for mice that were depleted of CD4⁺ T cells, indicating that late phase swelling was not due to type IV hypersensitivity (Fig. 8C). Finally, platelet depletion by i.p. administration of anti-CD41 24 hours prior to challenge also had no effect on ear swelling (Fig. 10).

It was only when antibody-deficient J_H mice were used that we saw complete abrogation of the late phase response (Fig. 8C). This result suggests that late phase ear swelling was due to immune complex-mediated type III hypersensitivity.

To assess this possibility, we performed immunohistochemistry on ears from Sensitized and Sensitized + Infected animals 3 hours after OVA challenge. Anti-mouse IgG conjugated to FITC was then used to visualize immune complex deposition in the ear tissue via confocal microscopy (Fig. 8D). Immune complexes were readily visible in both groups, confirming that late phase ear swelling in our model is due to type III

Figure 8. Protection against type III hypersensitivity

Immune complex-mediated ear swelling was significantly reduced Sensitized + Infected animals. **(A)** Ear swelling was measured by micrometer for 4 days following OVA and PBS challenge at 10 weeks post-infection. Data points for Sensitized and Sensitized + Infected groups were compared at each time point by the Mann Whitney test. **(B)** Ear swelling for uninfected OVA and PBS sensitized C57BL/6 control, IgE deficient, and mast cell deficient (W^{sh}) mice. Data is representative of two independent experiments composed of 3 animals per group. **(C)** Ear swelling for uninfected OVA and PBS sensitized BALB/c and J^H mice. Basophils and CD4 cells were depleted by administration of anti-CD200R and anti-CD4, respectively, 24 hours prior to challenge. Depletion was confirmed 72 hours post-challenge by flow cytometry. Data is representative of two independent experiments composed of three animals per group. Δ Thickness represents differences in ear thickness between PBS and OVA challenged ears (OVA-PBS) for each animal. **(D)** Visualization of immune complexes by immunohistochemistry 3 hours after PBS or OVA challenged by staining with anti-mouse IgG-FITC. Error bars represent \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



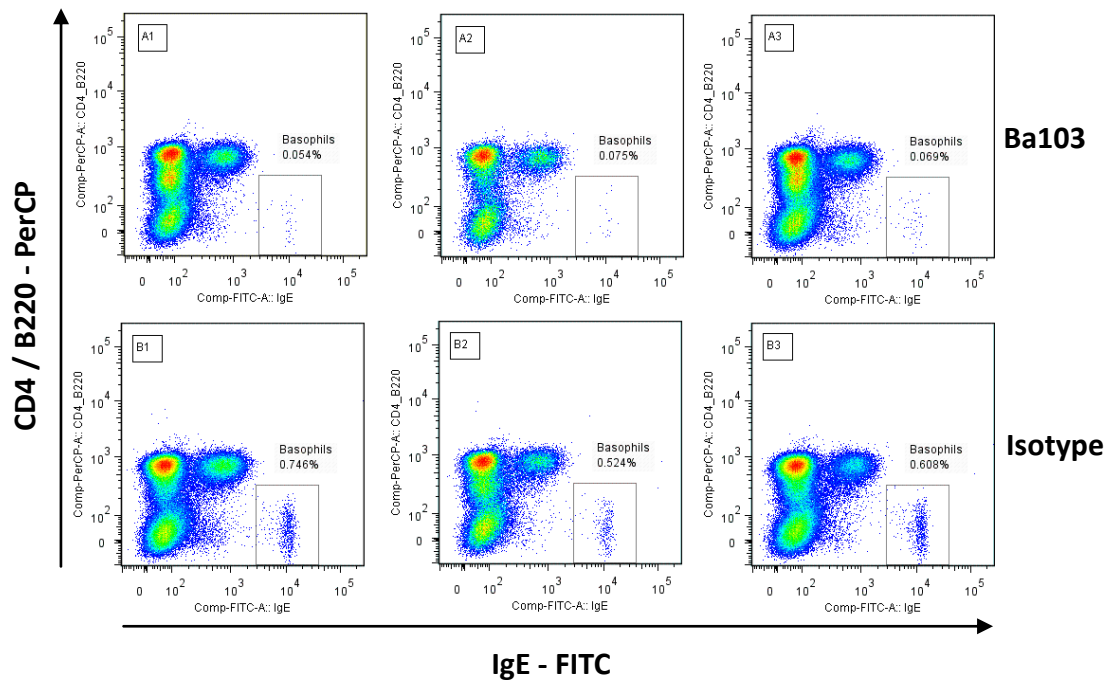


Figure 9. Basophil depletion with monoclonal antibody Ba103

Numbers of circulating basophils 4 days after treatment with Ba103 or isotype control antibody was assessed by flow cytometry. A terminal bleed was performed and cells were stained with anti-CD4 PerCP, anti-B220 PerCP, and anti-IgE FITC. Basophils are gated as CD4⁻B220⁻IgE⁺.

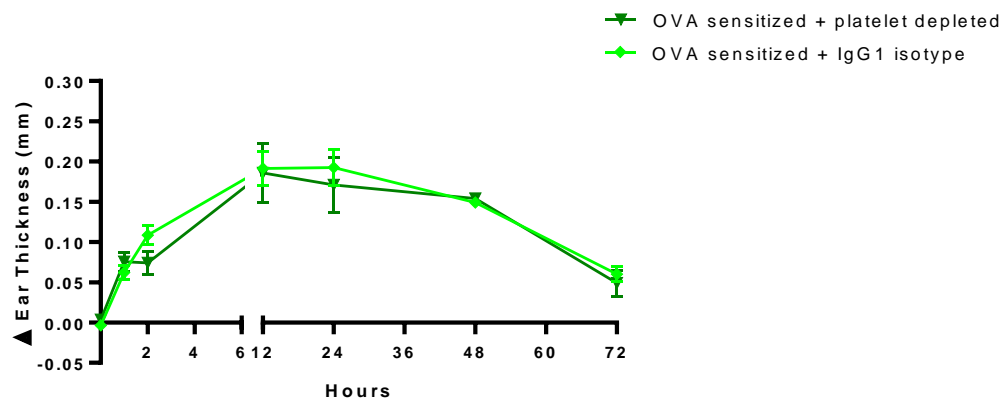


Figure 10. Ear thickness assay following platelet depletion

Ear thickness assay following platelet depletion. Platelets were depleted by i.p. injection of anti-CD41 24 hours prior to ear challenge. Depletion was confirmed by CBC analysis.

hypersensitivity. Indeed, the time course of the swelling observed in our model is most consistent with that of immune complex-mediated inflammation.

Infection enhances immune complex size and complement activation

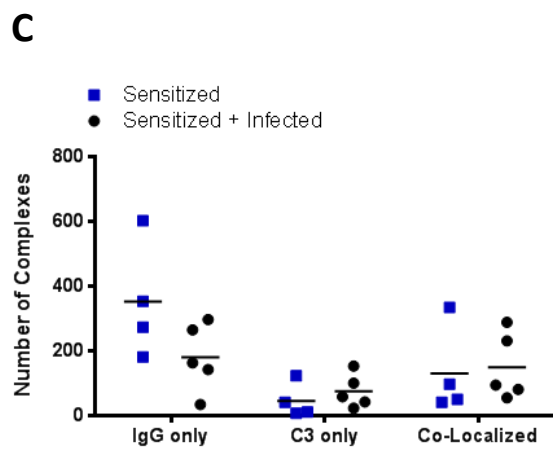
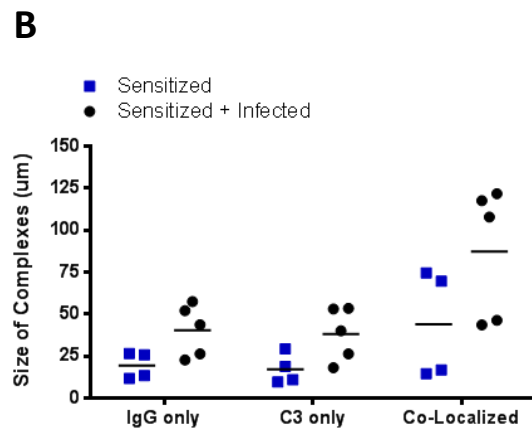
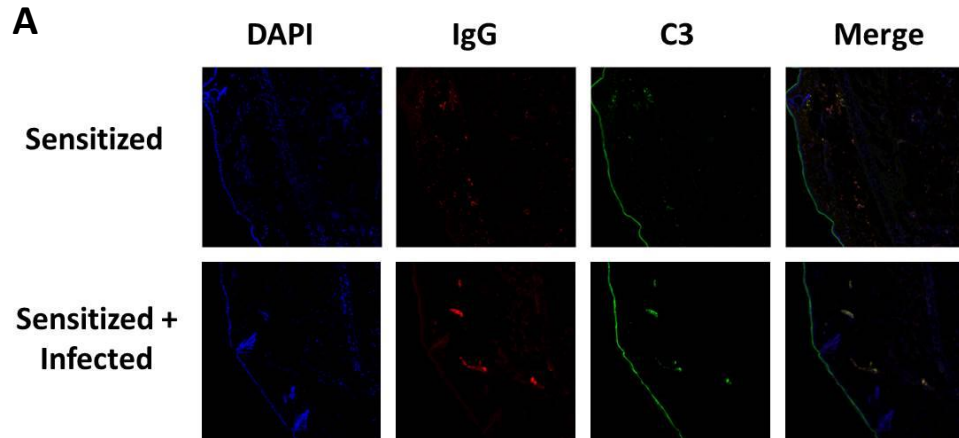
To evaluate whether infection induces changes in immune complex morphology, we performed immunohistochemistry for IgG and C3 and visualized immune complexes via confocal microscopy. Both Sensitized and Sensitized + Infected animals showed IgG, C3, and co-localized immune complex staining (Fig 11A), but there was no difference in the mean fluorescent intensity between groups (Fig. 12B). Furthermore, while there were no statistically significant differences, Sensitized + Infected animals tended to have larger complexes and fewer complexes composed of only IgG compared to Sensitized animals (Fig. 11B, C). Since larger complexes have the potential to be more pathogenic than smaller ones, these results suggest that the mechanism by which infection protects against ear swelling due to type III hypersensitivity likely occurs downstream of immune complex formation.

Chronic *L. sigmodontis* infection reduces levels of allergen-specific IgG

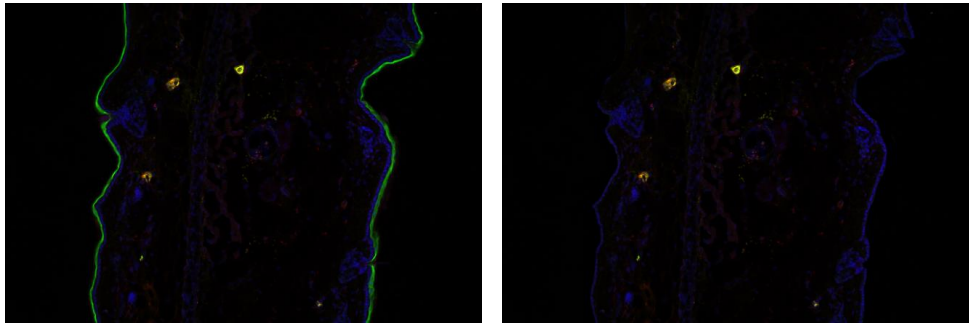
Total IgG, total IgG2a, OVA-specific IgG2a, and OVA-specific IgG1 antibody levels were measured by ELISA at 10 weeks post-infection. Sensitized + Infected animals had elevated levels of total IgG (Fig. 13A) but a significant reduction in total IgG2a (Fig. 13B), the predominant IgG subclass that participates in immune complex formation. Furthermore, OVA-specific IgG2a levels were also significantly lower in Sensitized + Infected animals compared to Sensitized animals. The reduction in OVA-specific IgG was not subclass-dependent, as OVA-specific IgG1, an anti-inflammatory

Figure 11. Immune complex morphology at 3 hours post challenge

Chronic *L. sigmodontis* infection alters immune complex morphology. Immunohistochemistry was performed on ears 3 hours after OVA challenge. Ear sections were stained with DAPI, anti-mouse IgG-Texas Red and anti-mouse C3-FITC to visualize immune complexes. Data are from two independent experiments with 2-3 animals per group. **(A)** Representative images taken with a Zeiss 710 confocal microscope at 10X with 50% zoom to show detail. For each animal, the entire ear was imaged and analyzed using the Puncta Analyzer plugin on ImageJ. **(B)** Average size of IgG only, C3 only, and co-localized complexes. **(C)** Number of IgG only, C3 only, and co-localized complexes.



A



B

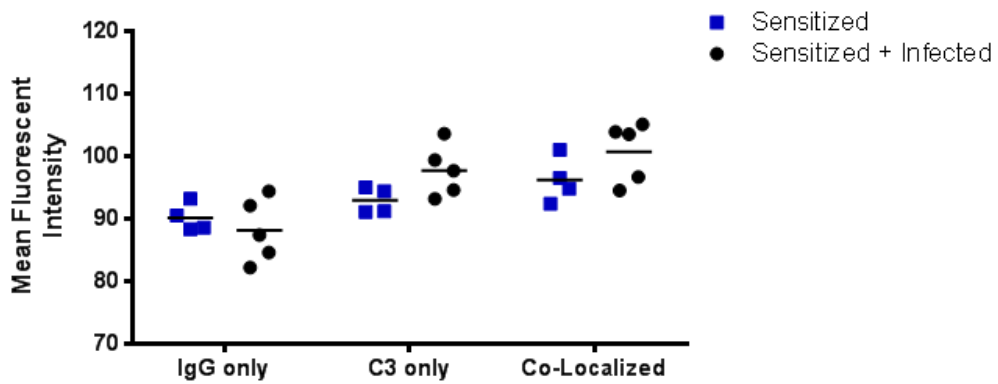
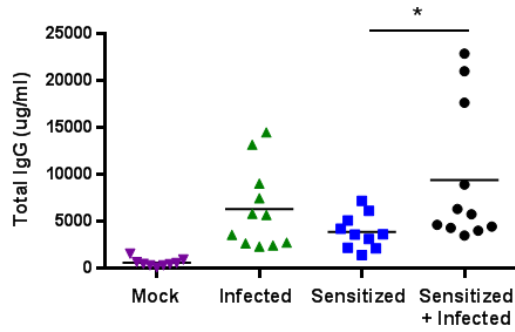
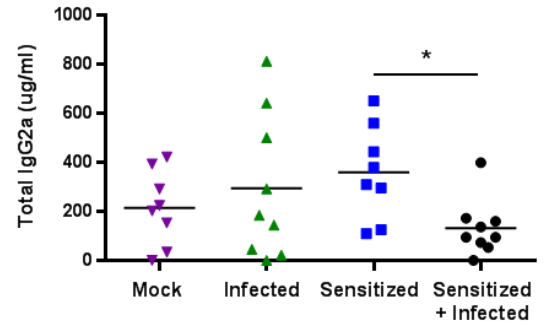
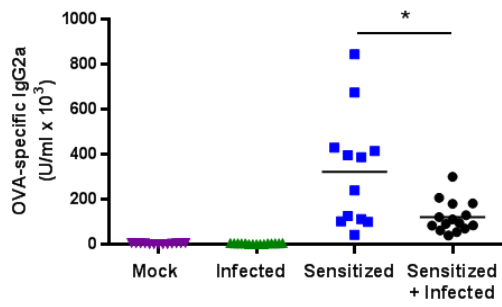
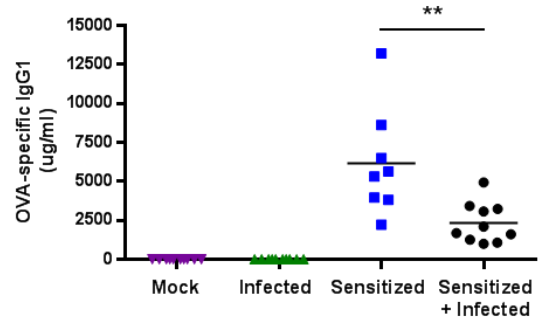
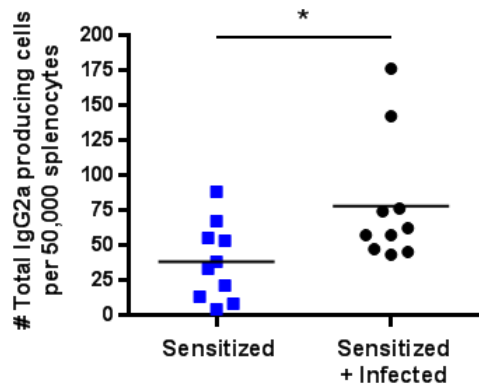
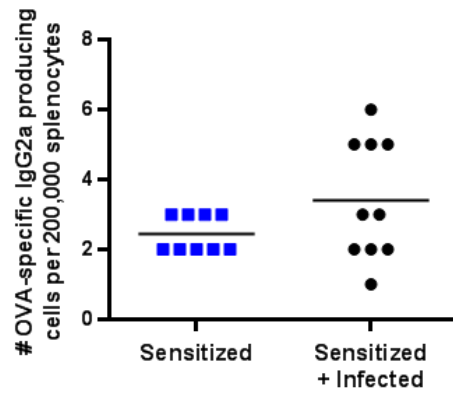


Figure 12. Masking of confocal images and mean fluorescent intensity

(A) Masking of non-specific C3 staining. Left panel represents image before masking, and right panel represents image after masking. (B) Mean fluorescent intensity of puncta was calculated by the Puncta Analyzer plugin on ImageJ.

Figure 13. Allergen-specific antibody levels

Chronically infected animals had reduced levels of allergen-specific antibodies. Levels of circulating total IgG (**A**), total IgG2a (**B**), OVA-specific IgG2a (**C**), and OVA-specific IgG1 were detected by ELISA at 10 weeks post-infection. ELISPOT assays for total IgG2a (**E**) and OVA-specific IgG2a (**F**) were performed on live, frozen splenocytes that were isolated from animals at the 10 week time point. Statistical differences between Sensitized and Sensitized + Infected groups were determined by the Mann Whitney test. * $p < 0.05$, ** $p < 0.01$.

A**B****C****D****E****F**

IgG subclass associated with tolerance (165), was also lower in Sensitized + Infected animals compared to the Sensitized group (Fig.13D).

To ascertain whether the reduction in OVA-specific IgG2a was due to a defect in antibody production, ELISPOT assays were performed. Sensitized + Infected animals had higher numbers of total IgG2a (Fig. 13E) and OVA-specific IgG2a (Fig. 13F) producing cells than Sensitized animals. These data indicate that antibody production is not compromised during chronic infection, suggesting that the reduction in OVA-specific antibody levels in infected animals may be due to increased antibody catabolism.

Infection significantly reduces pathology at 24 hours post challenge

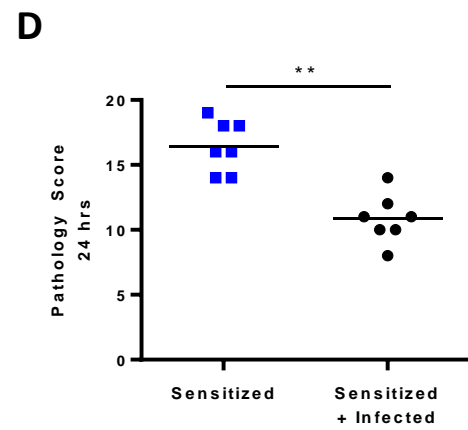
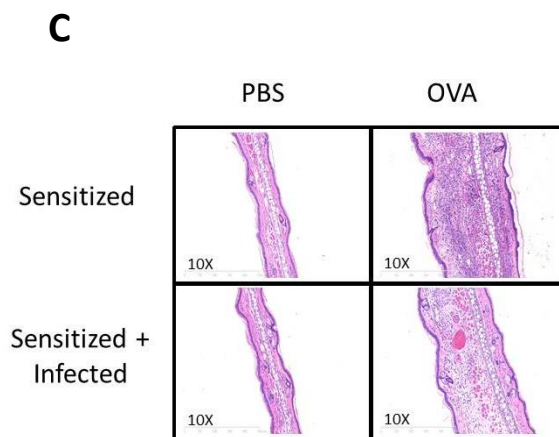
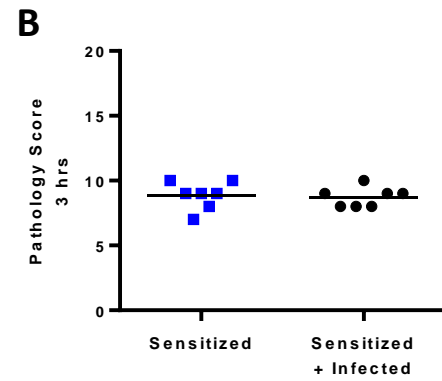
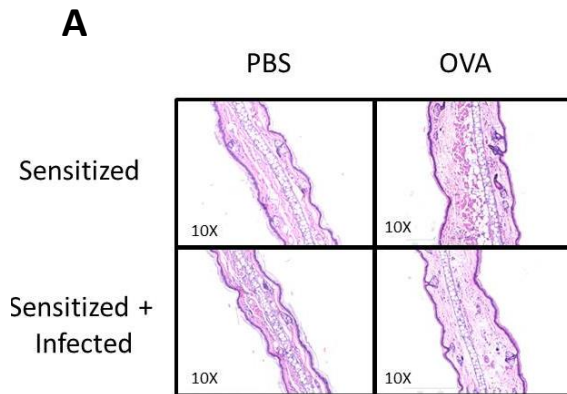
Ear pathology was assessed by H&E stain at 3 hours post-challenge, the time point at which immune complexes were visualized by immunohistochemistry (Fig. 14A), and 24 hours post challenge, the time point of maximal ear swelling. A blinded pathologist scored the tissue sections based on severity and focality of edema, hemorrhage, necrosis, and cellularity. Scores for OVA challenged ears were comparable between Sensitized + Infected and Sensitized animals at the 3 hour time point (Fig. 14B). However, at 24 hours post challenge, ears of Sensitized animals exhibited marked cellular infiltration. As seen in figure 14C, cellular infiltration was also present in Sensitized + Infected animals, but to a much lower degree. Sensitized + Infected animals had a significant reduction in pathology score at 24 hours post challenge (Fig. 14D), confirming the protective effect observed when performing the ear thickness assay.

Infection reduces neutrophil and macrophage recruitment following OVA challenge

After observing reduced swelling and cellularity in Sensitized + Infected animals, we quantified the specific cell types recruited to the ear tissue after allergen exposure.

Figure 14. Ear histology at 3 and 24 hours post challenge

Sensitized + Infected animals exhibited reduced ear pathology 24 hours post-challenge. **(A)** H&E stain of ear tissue at 3 hours post-challenge, the time point at which immune complexes were detected by immunohistochemistry. **(B)** Pathology score for 3 hour time point. Four parameters were measured (hemorrhaging, edema, necrosis, and cellularity) on the basis of severity (0-absent, 1-mild, 2-moderate, or 3-severe) and focality (0-absent, 1-focal, 2-intermediate, and 3-diffuse) for a total maximum score of 24. **(C)** H&E stain of ear tissue at 24 hours post-challenge, the time point at which there was the greatest difference in ear swelling between groups. **(D)** Pathology score for 24 hour time point. Significant differences between groups were analyzed by the Mann Whitney test. ** $p < 0.01$.



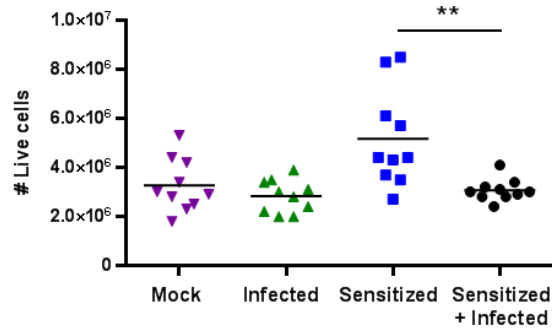
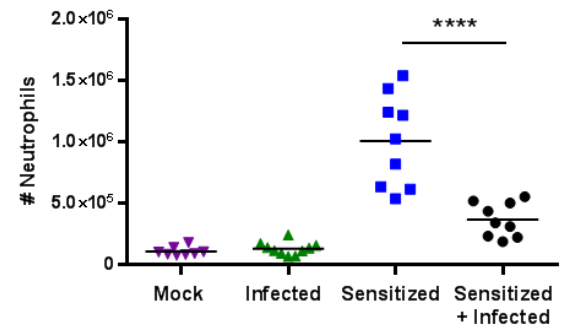
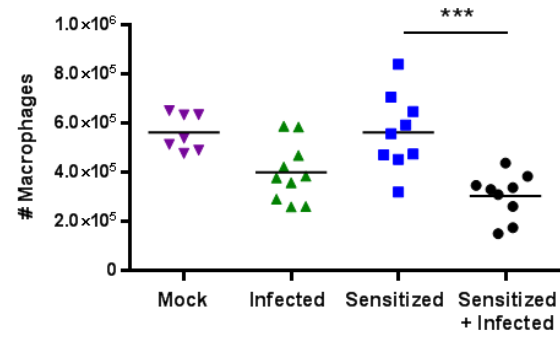
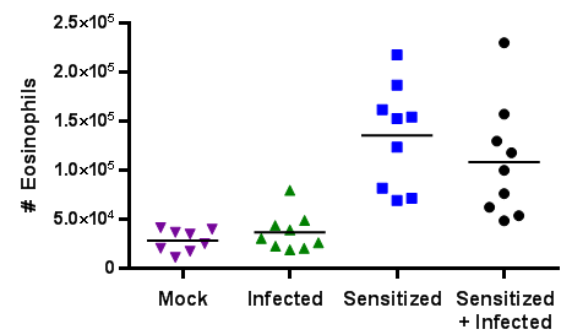
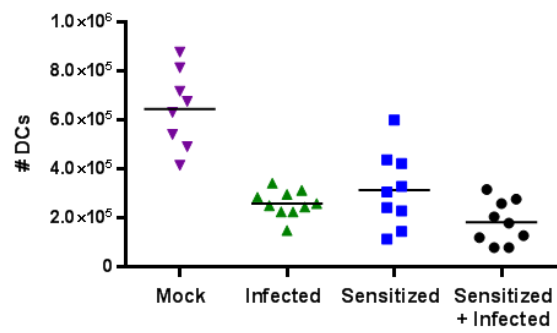
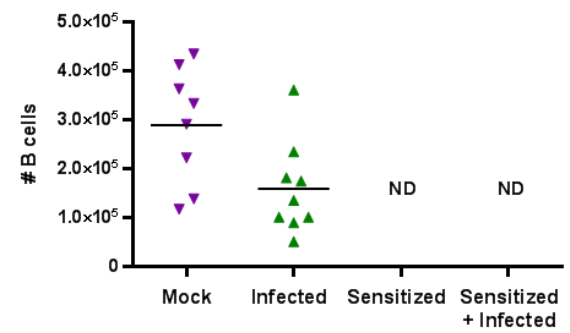
A single cell suspension of ear tissue was prepared 24 hours after OVA challenge. Cells were enumerated on a hemacytometer and stained for flow cytometry to determine the cell types present. The number of live cells in the ear tissue was significantly reduced in Sensitized + Infected animals compared to Sensitized animals (Fig. 15A); however there was no difference in the number (Fig. 16A) or percentage (Fig. 16B) of dead cells between groups.

Neutrophils are the primary cell type that participates in immune complex-mediated inflammation, and the severity of type III hypersensitivity reactions can be correlated to the number of neutrophils present (103). We found a significant reduction in the number of neutrophils recruited in Sensitized + Infected animals following OVA challenge (Fig. 15B). Macrophages were also present in lower numbers in infected animals (Fig. 15C). There was no difference in the numbers of eosinophils (Fig. 15D) or dendritic cells (Fig. 15E) between Sensitized and Sensitized + Infected groups. B cell numbers were below the limit of detection for Sensitized and Sensitized + Infected mice (Fig. 15F).

To ensure that differences in cellular infiltration were not due to reduced availability of circulating white blood cells, we performed a CBC analysis on animals 10 weeks post-infection (Fig. 17). Sensitized + Infected animals had equal or higher numbers of circulating lymphocytes and granulocytes, including neutrophils and monocytes. These data indicate that helminth infection suppresses neutrophil and monocyte recruitment to immune complexes.

Figure 15. Cell recruitment to the ear 24 hours post challenge

Sensitized + Infected animals showed impaired neutrophil and macrophage recruitment to the ear following allergen challenge. **(A)** At 24 hours after OVA challenge, a single cell suspension of ear tissue was created and live cell counts were obtained by hemacytometer. Flow cytometry was then performed on the cell suspensions to determine the number of **(B)** neutrophils [F4/80⁻Ly6G⁺], **(C)** macrophages [F4/80⁺], **(D)** eosinophils [CD11c⁻CD45⁺SiglecF⁺], **(E)** dendritic cells [CD11c⁺], and **(F)** B cells [CD19⁺] present. Gates were determined using fluorescence minus one controls. Significant differences between Sensitized and Sensitized + Infected groups were calculated using the Mann Whitney test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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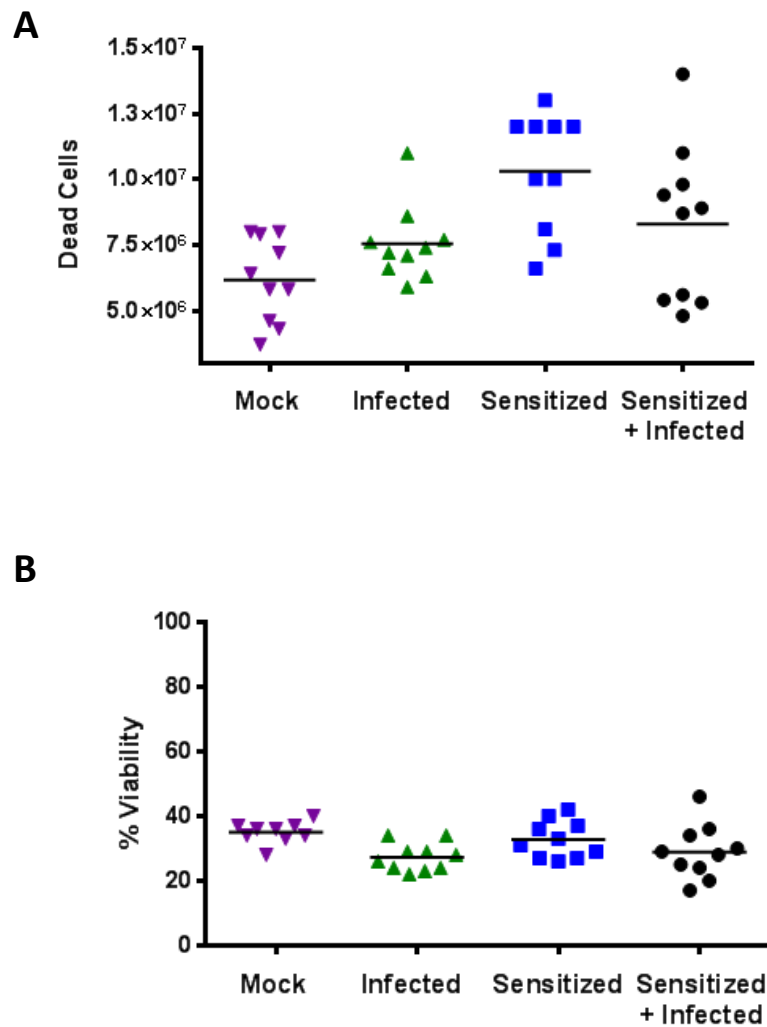


Figure 16. Number of dead cells and cell viability 24 hours post-challenge

At 24 hours after OVA challenge, a single cell suspension ear tissue was prepared. The number of dead cells (**A**) and cell viability (**B**) was obtained by hemacytometer.

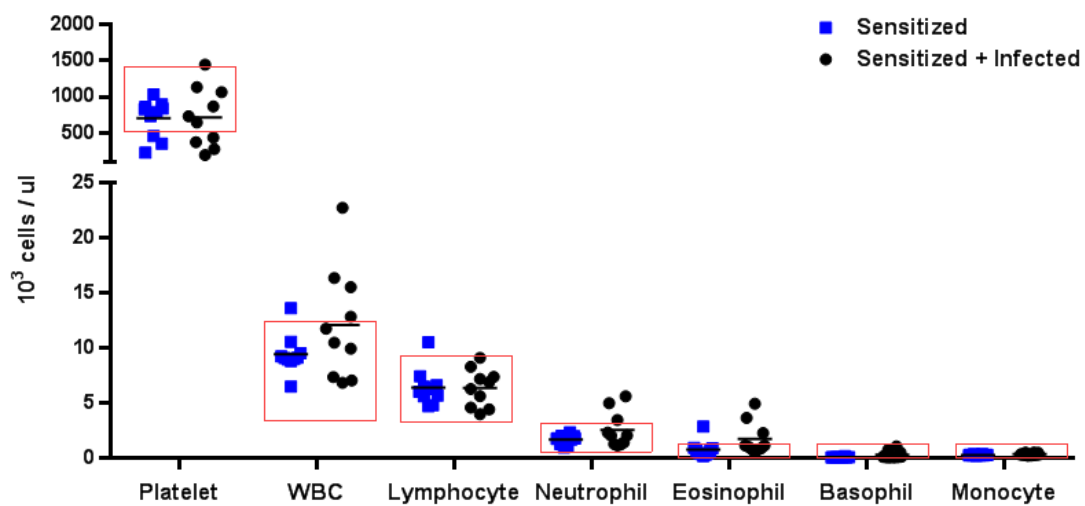


Figure 17. Number of circulating leukocytes 10 weeks post-infection.

Numbers of circulating cells per μl blood, as determined by CBC analysis. Boxes indicate normal range for each cell type. There was no different between Sensitized and Sensitized + Infected animals.

Infection suppresses neutrophil-specific chemokine production by eosinophils

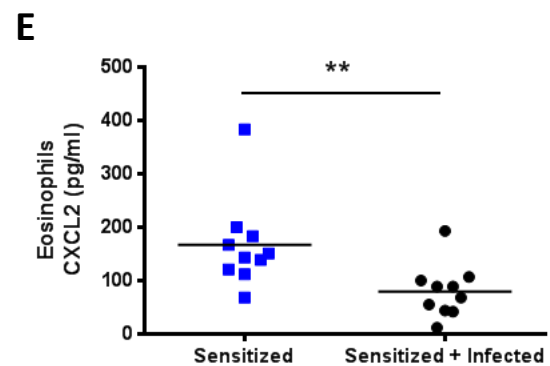
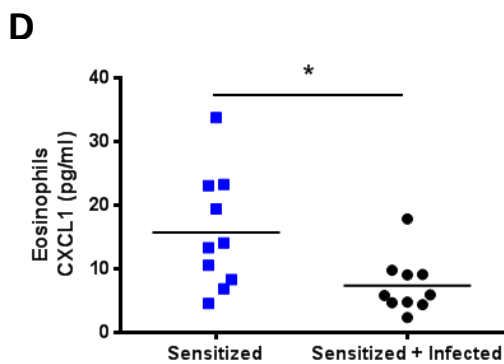
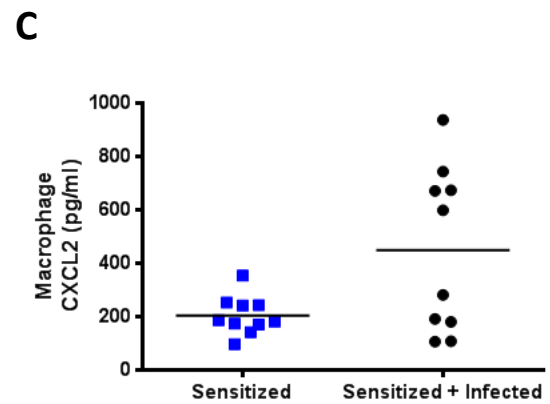
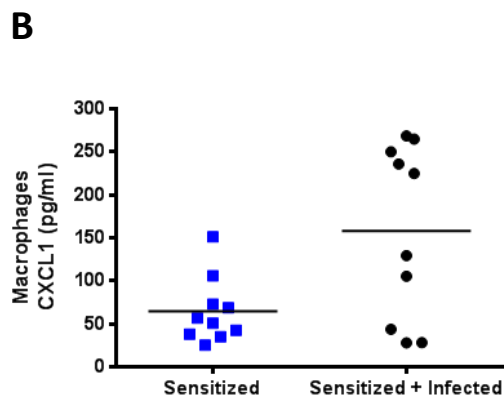
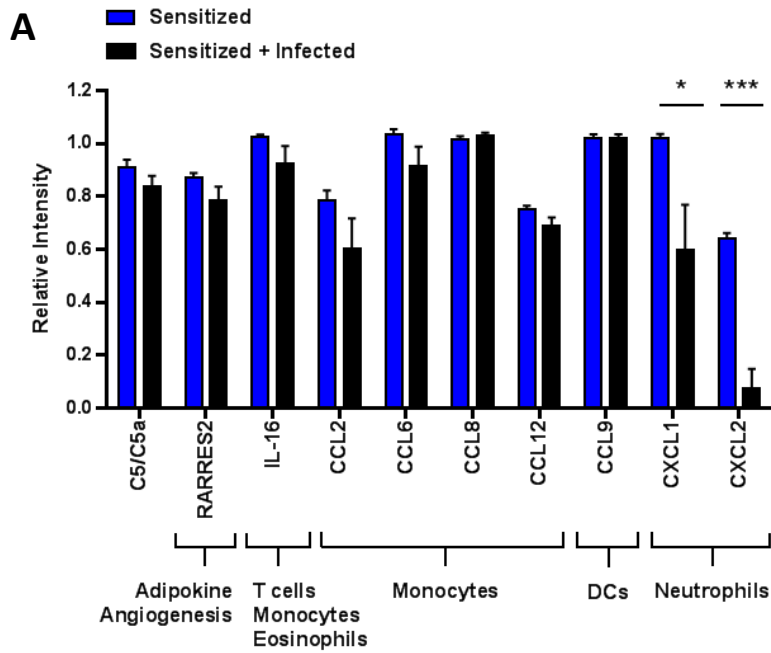
To investigate the underlying mechanism driving impaired cell recruitment in Sensitized + Infected animals, we used a chemokine profiler array to measure relative concentrations of chemokines present in the ear 6 hours after OVA challenge. Of the 25 chemokines assayed by the array, 10 were present at detectable levels in the ear tissue 6 hours after challenge. Of these, only neutrophil-specific chemokines CXCL1 (KC) and CXCL2 (MIP-2) were significantly reduced in Sensitized + Infected animals (Fig. 18A). These data correlate with the reduced neutrophil recruitment observed in Figure 15B. The remaining chemokines, including monocyte-specific chemokines CCL2, CCL6, and CCL12, tended to be only slightly lower in Sensitized + Infected animals compared to Sensitized animals. While other time points may have elicited a more robust difference between groups, the slight reduction in multiple monocyte-specific chemokines in infected animals may have had a cumulative effect on macrophage recruitment following OVA challenge (Fig. 15C).

The two principal cell types that release CXCL1 and CXCL2 in response to immune complex activation are macrophages and eosinophils. To test the effect chronic helminth infection has on chemokine production by these cells, we next stimulated enriched populations of macrophages and eosinophils from Sensitized and Sensitized + Infected mice with immune complexes *in vitro*. Immune complexes were generated by combining polyclonal rabbit anti-OVA IgG antibody with OVA at a ratio of 100:1. This resulted in the formation of large, precipitating immune complexes after a 30 minute incubation at 37°C.

As seen in Figures 18B and 18C, CXCL1 and CXCL2 production by macrophages was greater in Sensitized + Infected animals than Sensitized animals,

Figure 18. Neutrophil-specific chemokine production

Neutrophil-specific chemokine production by eosinophils was reduced in Sensitized + Infected animals. **(A)** Chemokine array performed on ear tissue 6 hours after OVA challenge. Both ears were challenged with OVA and pooled for each animal. Tissues were homogenized with FastPrep lysing matrix D beads in the presence of protease inhibitors, and supernatants assayed for the presence of 25 distinct chemokines. To determine the cell types responsible for neutrophil-specific chemokine production, macrophages were isolated from the peritoneal cavity and an eosinophil-enriched cell fraction was derived from splenocytes. Cells were stimulated with immune complexes for 6 hours *in vitro* and supernatants were harvested to assess macrophage production of CXCL1 **(B)** and CXCL2 **(C)**, and eosinophil production of CXCL1 **(D)** and CXCL2 **(E)** by ELISA. Error bars represent \pm SEM. Significant differences between groups were analyzed by the Mann Whitney test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



though the difference was not statistically significant. In contrast, eosinophil production of CXCL1 (Fig. 18D) and CXCL2 (Fig. 18E) was markedly reduced in chronically infected mice compared to Sensitized mice (P values < 0.05 and <0.01, respectively). The suppression of CXCL2 production by eosinophils in Sensitized + Infected animals was more robust than suppression of CXCL1 production, consistent with the data obtained by the chemokine array (Fig. 18A). These results suggest that the suppression of neutrophil-specific chemokine production in chronically infected mice may be due to suppression of eosinophil function.

Eosinophil expression of FcγRI is suppressed in chronically infected animals

To understand why eosinophils from chronically infected animals produced lower levels of neutrophil-specific chemokines, we measured the surface expression of activating Fc gamma receptors I (FcγRI) and III (FcγRIII), and inhibitory Fc gamma receptor II (FcγRII) on multiple cell types by flow cytometry.

Sensitized + Infected animals showed a significant reduction in the percentage of eosinophils expressing FcγRI compared to Sensitized animals (Fig. 19A). There was no difference in the percentage of eosinophils expressing the other activating receptor FcγRIII, or the inhibitory receptor FcγRII. Furthermore, there was no difference between groups with regard to the percentage of macrophages (Fig. 19B), neutrophils (Fig. 19C), dendritic cells (Fig. 19D) or B cells (Fig. 19E) expressing FcγRI, FcγRII, or FcγRIII. The only appreciable difference in receptor expression levels on the surface of any cell type was a reduction in FcγRI on eosinophils, as indicated by MFI values (Fig. 20). Notably, the absence of neutrophils expressing FcγRI or FcγRIII (Fig. 19C) signifies that

Figure 19. Cell expression of FcγRI, FcγRII, and FcγRIII

Infection results in decreased percentage of eosinophils expressing FcγRI. Flow cytometry was used to assess the percentage of cells expressing activating Fc gamma receptors I and III, and inhibitory Fc gamma receptor II. Single cell suspensions of live splenocytes were first gated as **(A)** eosinophils [CD11c⁻CD45⁺SiglecF⁺], **(B)** macrophages [F4/80⁺], **(C)** neutrophils [F4/80⁻Ly6G⁺], **(D)** dendritic cells [CD11c⁺], or **(E)** B cells [CD19⁺]. Each cell type was then gated on the basis of FcγRI [CD64⁺], FcγRIII [CD16⁺] and FcγRII [CD32⁺] positivity using fluorescence minus one (FMO) controls. Error bars represent \pm SEM. Significant differences between groups were analyzed by the Mann Whitney test. **** $p < 0.0001$.

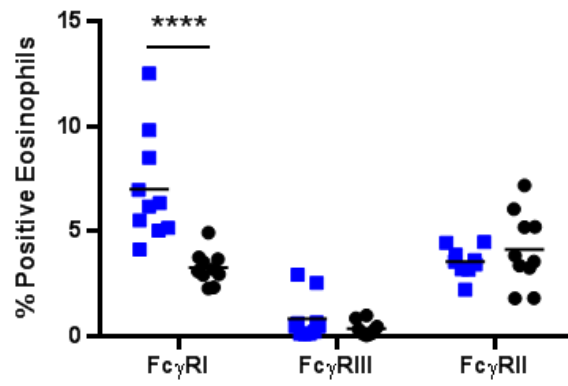
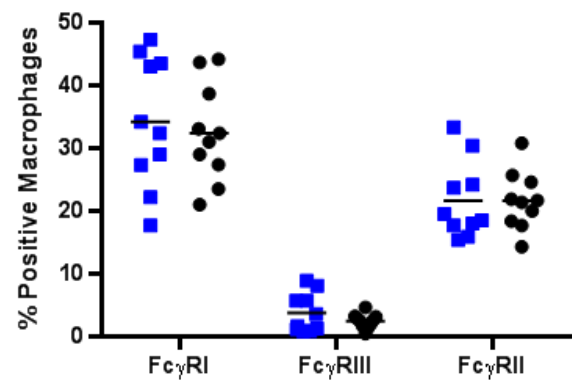
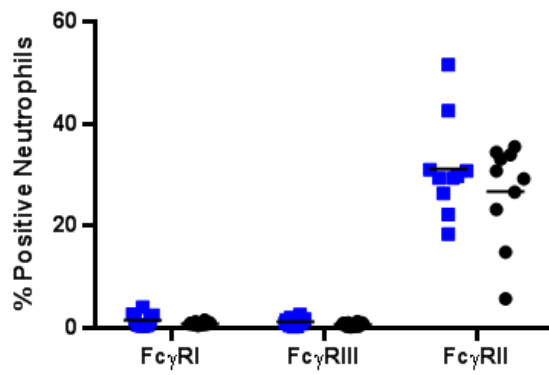
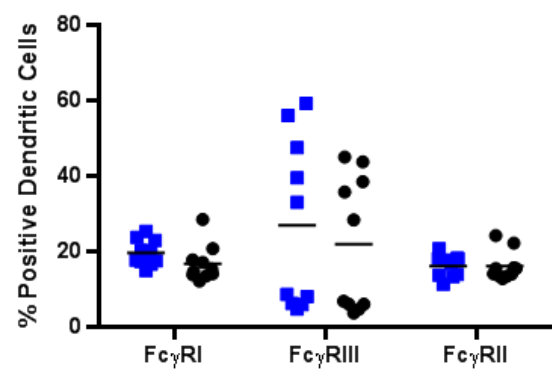
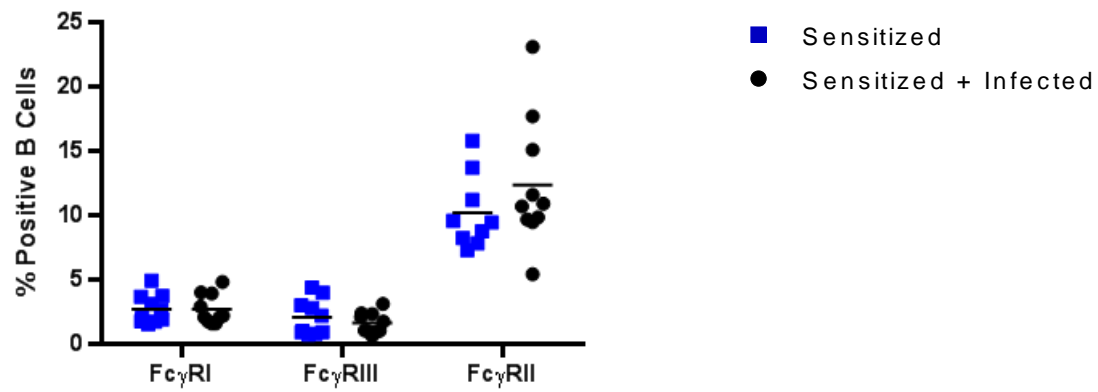
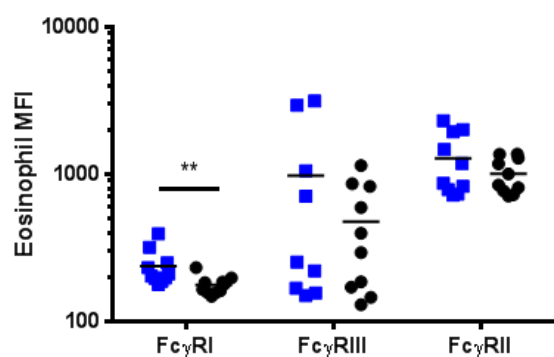
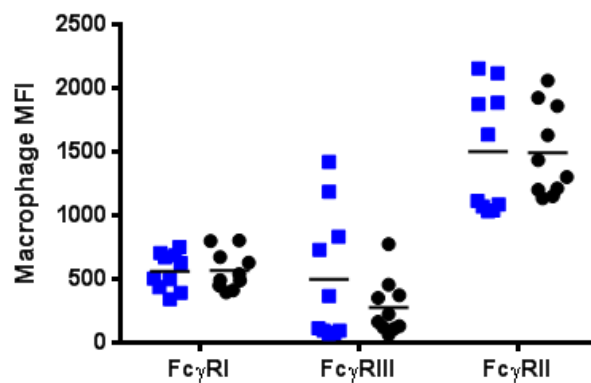
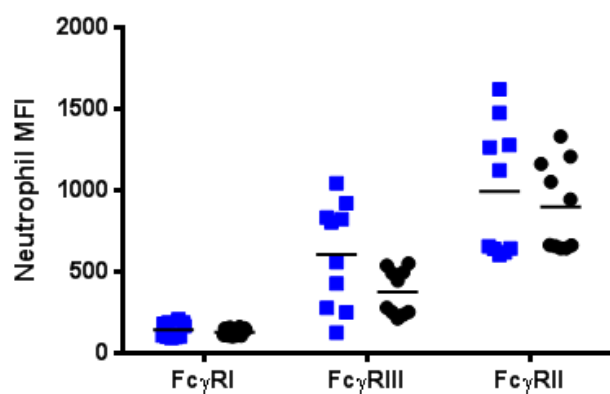
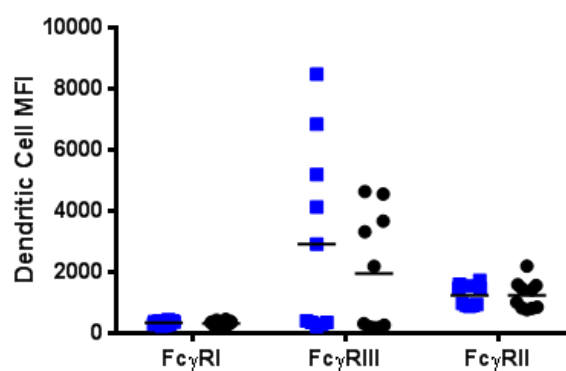
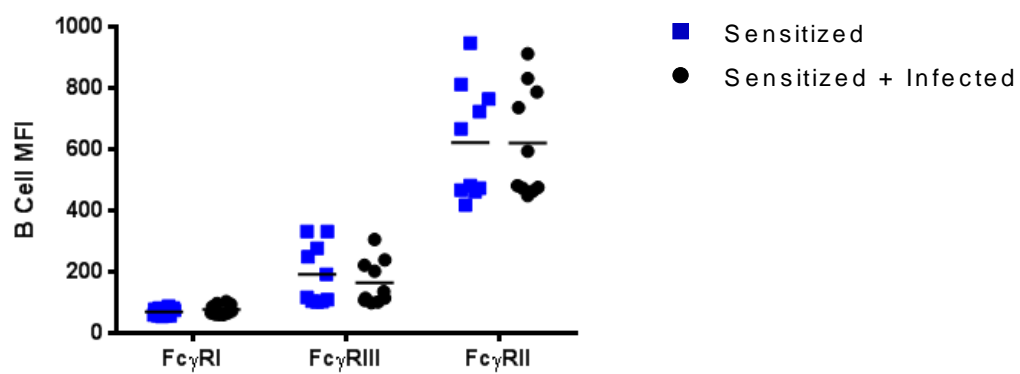
A**B****C****D****E**

Figure 20. Mean fluorescent intensity of FcγRI, FcγRII, and FcγRIII

MFI for FcγRI, FcγRII, and FcγRIII on eosinophils (**A**), macrophages (**B**), neutrophils (**C**), dendritic cells (**D**), and B cells (**E**).

A**B****C****D****E**

neutrophils are not likely to be major contributors to the chemokine production observed from the eosinophil-enriched cell fraction (Figure 18).

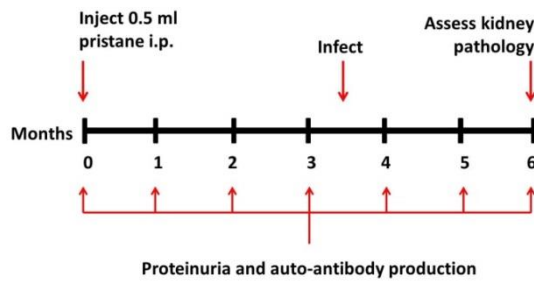
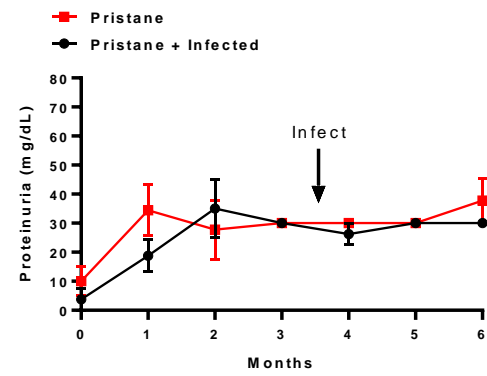
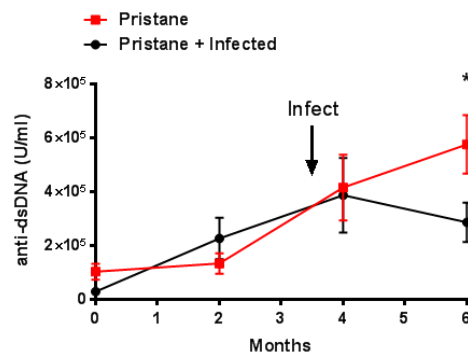
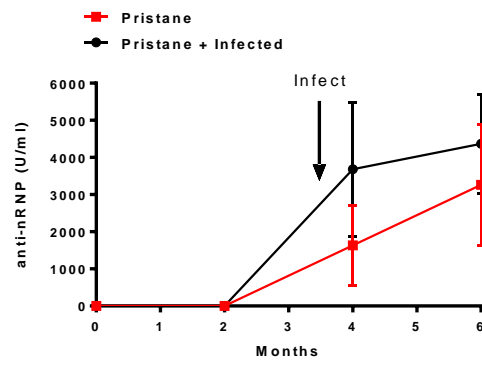
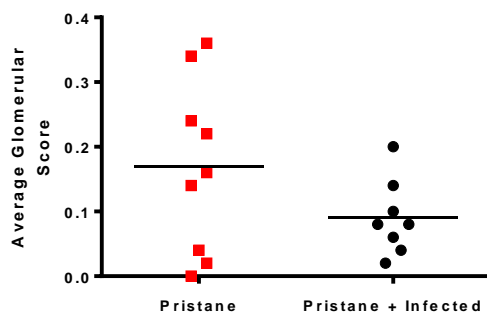
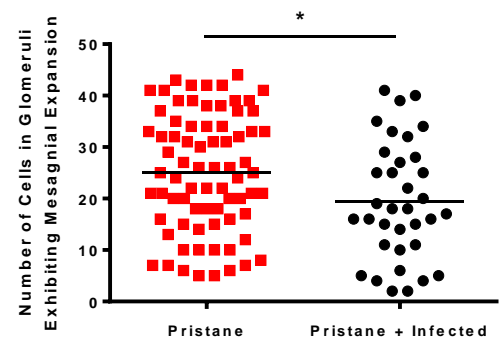
Infection reduces autoantibody levels and mesangial expansion in mice with SLE.

To assess whether chronic infection protects against type III hypersensitivity diseases other than the local Arthus reaction model we had been utilizing, we tested whether *L. sigmodontis* infection is protective in the pristane/BALB/c murine model of systemic lupus erythematosus (SLE). In this model, glomerular disease, especially mesangial proliferation, occurs as a result of immune complex-mediated inflammation. For this experiment, we injected pristane i.p. and monitored proteinuria and autoantibody levels on a monthly basis. Fourteen weeks after pristane treatment, well after the autoimmune process had been established, mice were infected with *L. sigmodontis*. Ten weeks later (24 weeks after pristane treatment) mice were euthanized and kidney pathology was scored (Fig. 21A).

Proteinuria peaked and then began to plateau 6 weeks prior to injection of *L. sigmodontis* worms and was not affected by infection (Fig. 21B). While levels of anti-dsDNA were significantly lower in chronically infected animals at the 10 week time point (Fig. 21C), anti-nRNP levels were the same for both groups (Fig. 21D). Kidney pathology, as indicated by average glomerular score, was decreased in chronically infected mice (Fig. 21E). While the decrease in kidney pathology score was not statistically significant, the number of cells present in glomeruli exhibiting mesangial expansion was significantly reduced in chronically infected animals (Fig. 21F).

Figure 21. Helminth infection and protection against SLE

Infection reduces levels of anti-dsDNA autoantibodies and mesangial expansion in mice with SLE. **(A)** Experimental design. **(B)** Proteinuria levels were monitored monthly with Albustix reagent strips. Circulating levels of autoantibodies anti-dsDNA **(C)** and anti-nRNP **(D)** were measured by ELISA. Fifty glomeruli per mouse were scored for mesangial expansion, endocapillary proliferation, capillaritis, and crescents **(E)**. Cells were then counted in glomeruli that exhibited mesangial expansion **(F)**.

A**B****C****D****E****F**

DISCUSSION

In this study, we found that chronic helminth infection with the filarial parasite *L. sigmodontis* protects against type III, but not type I, hypersensitivity in a murine ear challenge model. Protection was associated with reduced neutrophil influx into the ear, decreased local levels of the CXCL1 and CXCL2 neutrophil chemokines, and diminished production of these chemokines by eosinophils in response to immune complex stimulation. Additionally, we found that chronic filarial infection exerts a therapeutic effect on established lupus nephritis, as infected animals exhibited reduced levels of anti-dsDNA and decreased mesangial expansion.

In our first experiment, we evaluated whether chronic *L. sigmodontis* infection protects against type I hypersensitivity in mice previously sensitized against ovalbumin (OVA). Even though 10 weeks of infection resulted in lower OVA-specific IgE levels, reduced basophil activation in response to OVA stimulation, and decreased numbers of degranulated tissue mast cells after intradermal OVA challenge, no clinical protection against immediate local anaphylaxis was observed using an Evans Blue assay to measure changes in vascular permeability.

Although numerous studies have shown helminths protect against allergy when given prior to sensitization, the few animal studies in which infection was established after sensitization have produced mixed results (96; 160; 239; 241). Given that *L. sigmodontis* was shown to be beneficial when given prior to allergic sensitization (47), it is likely that helminth infections are more effective at blunting sensitization than preventing symptoms after sensitization has occurred. Thus, these results support the conclusion that helminth infections may not readily protect against immediate hypersensitivity reactions in previously sensitized individuals. Indeed, to date, the only

two clinical studies that have prospectively tested whether helminth infections can be given to protect against allergic disease have been negative (15; 57).

That said, the results of our experiment do not completely rule out the possibility that helminth infections can have beneficial effects on individuals with established immediate hypersensitivities. Indeed, the major immunologic correlates of type I hypersensitivity (allergen-specific IgE as well as basophil and mast cell degranulation in response to allergen) were decreased in the setting of *L. sigmodontis* infection. We speculate that these decreases did not translate into clinical protection because either A) they were not of sufficient magnitude for the dose of allergen given, or B) infection did not occur for a long enough period of time. With regards to magnitude of change, the decrease in OVA specific-IgE may not have been great enough to decrease mast cell sensitivity to IgE cross-linking. In terms of duration, a 10 week infection may not be long enough to substantially alter the repertoire of IgE antibodies on the surface of tissue-resident mast cells. Because these are long-lived cells with slow turnover at tissue sites (106), it is possible that decreases in circulating levels of allergen-specific IgE may take months to result in substantial reductions in mast cell sensitivity to allergen. Testing whether a longer exposure to helminths can provide clinical protection against immediate hypersensitivity will be the focus of future studies.

Interestingly, while we failed to observe clinical protection against type I hypersensitivity, we did find significant protection against late ear swelling due to type III hypersensitivity. Type III hypersensitivity is driven by immune complex deposition and is a major pathogenic mechanism for diseases such as SLE (177), serum sickness (110), and post-streptococcal glomerulonephritis (63). To our knowledge, this is the first

study to specifically demonstrate that helminth infection can protect against immune complex-mediated hypersensitivity.

The mechanism by which *L. sigmodontis* protects against type III hypersensitivity in our model is likely downstream of immune complex formation. When antibody or antigen is in excess, small immune complexes form. At the equivalence point, when the concentrations of antibody and antigen are similar, large immune complexes form. Sensitized animals had high levels of OVA-specific IgG and small immune complexes, indicating that there was a state of antibody excess. Infected animals demonstrated reduced levels of OVA-specific IgG2a, yet their immune complexes were larger than in uninfected mice. Therefore, decreases in OVA-specific IgG were likely driving the antibody to antigen ratio closer to the equivalence point, and thus larger immune complexes formed (Fig. 11). Large immune complexes have the potential to induce more inflammation than small immune complexes. The fact that the large complexes found in Sensitized + Infected mice were associated with less inflammation than the smaller complexes in Sensitized mice suggests protection occurs downstream of complex formation.

While immune complexes were visualized at 3 hours post-challenge, the reduction in pathology and cellular infiltration was observed at 24 hours post-challenge (Fig. 14). This difference in pathology was associated with fewer neutrophils and macrophages trafficking to the site of allergen challenge (Fig. 15).

To determine why Sensitized + Infected animals had fewer cells recruited to the ear, we monitored chemokine production after OVA challenge and *in vitro* immune complex stimulation. We observed a reduction in the neutrophil attractant chemokines

CXCL1 and CXCL2, and noted that eosinophils were specifically impaired in their ability to secrete these chemokines (Fig. 18). Conversely, macrophage production of chemokines was not suppressed, and in some animals was even exacerbated. Although macrophages are considered to be the primary cell type responsible for neutrophil-specific chemokine production, eosinophils have the capacity to produce large volumes of these chemokines in response to stimulation (191).

In addition to neutrophils, the number of macrophages present in the ear tissue was significantly suppressed in chronically infected animals (Fig 15), although this was not reflected by a substantial suppression of monocyte-specific chemokines produced 6 hours post challenge (Fig. 18). While assessing chemokine production at other time points may reveal more distinct differences, reduced monocyte recruitment may have been due to an impaired ability of monocytes to respond to chemokines, rather than reduced chemokine production. Indeed, macrophages and neutrophils from mice infected with *Echinococcus multilocularis* lost their ability to migrate in response to stimulation with worm antigen or endotoxin-activated mouse serum (6). Interestingly, this inhibitory effect was observed for chronic, but not acute, infection. Furthermore, taeniaestatin, a protease inhibitor isolated from *Taenia taeniaeformis*, prevented neutrophil chemotaxis in response to C5a (123).

The activation of cells by immune complexes involves ligation of activating (FcγRI and FcγRIII) and inhibitory (FcγRII) receptors. The ratio of activating and inhibitory receptor binding plays an integral role in determining whether cellular responses will be pro-inflammatory or anti-inflammatory. While the number of circulating eosinophils was not decreased in Sensitized + Infected mice (Fig. 17), the

percentage of eosinophils expressing Fc γ RI was reduced by 50% compared to Sensitized animals (Fig. 19). Furthermore, the amount of Fc γ RI expressed on the surface of eosinophils was also reduced, as indicated by MFI data (Fig. 20). This suggests that chronic *L. sigmodontis* infection lowers the propensity for eosinophils to express Fc γ RI, which in turn reduces the ability of this cell type to produce chemokines upon immune complex stimulation.

In addition to establishing systemic immunoregulatory networks, helminths release immune-modulatory factors in the form of excretory-secretory product. Because only eosinophils displayed a marked difference in Fc γ RI expression, it is possible that *L. sigmodontis* worms release factors that directly suppress eosinophils. Previous studies have already shown that worm products are associated with altered eosinophil function and reduced chemotaxis (168; 193; 202). The selective suppression of Fc γ RI expression on eosinophils during the course of infection would be advantageous to the parasite, as eosinophils are known to mediate worm clearance through antibody-dependent cell-mediated cytotoxicity (ADCC) (111).

The fact that eosinophils were the only cell type to display decreased Fc γ RI expression and chemokine production suggests that we may be able to develop medications that specifically suppress eosinophil function. Because eosinophils exhibited enhanced immunoregulation during infection, worm-mediated therapies may be particularly well-suited for eosinophil-driven diseases, such as eosinophilic esophagitis.

Interestingly, even though they did not play a mechanistic role in protecting mice against the allergic responses evaluated in this study, both OVA-specific IgG and IgE levels were lower in the setting of chronic *L. sigmodontis* infection. Our SLE experiments

indicate that infection also has the capacity to suppress levels of circulating autoantibodies. The reduction in allergen-specific and autoantibody levels is an interesting phenomenon, as other groups have reported similar results when infection is given prior to sensitization (17; 47; 180). In contrast to reductions in antibodies specific to environmental or self-antigens, levels of *L. sigmodontis*-specific and non-specific antibodies rise throughout the course of infection (223). Indeed, our ELISA and ELISPOT data (Fig. 13) confirm that infected animals are not compromised in their ability to elicit humoral immune responses. We therefore hypothesize that the decrease in allergen-specific antibodies is due to increased antibody catabolism. We speculate that the host increases antibody catabolism in an effort to counterbalance the high levels of antibodies produced during infection. To this effect, infection may be providing an endogenous source of antibodies that produces beneficial effects similar to intravenous immunoglobulin administration. To test this, future studies would likely investigate the role of FcRn for IgG catabolism, or CD23 for IgE catabolism (31), during the course of infection.

With regards to type III hypersensitivity, patients with SLE are far more common than patients suffering from Arthus reactions. Furthermore, SLE is a chronic disease that has limited treatment options. Because of the promising results observed with our cutaneous model of immune complex-mediated inflammation, we sought to determine whether *L. sigmodontis* infection could also be used as a therapeutic for SLE.

Our data indicate that when infection is given after SLE is already established, there is a reduction in kidney pathology, as indicated by the decreased number of cells in glomeruli exhibiting mesangial expansion (Fig. 21). These data agree with previously

published studies showing that worms and worm products can suppress cellular proliferation (7; 8; 82). Finding that helminths can protect against markers of SLE disease after immune complex formation has occurred suggests that we may potentially be able to develop novel therapies for immune complex-mediated diseases. Because *L. sigmodontis* can only establish chronic, patent infection in BALB/c mice, future studies would need to test the use of parasite antigen with other murine models of SLE, including MRL/*lpr* mice or F1 hybrids of NZW and NZB mouse strains.

In summary, we have shown that chronic infection with the rodent filarial worm *L. sigmodontis* protects the host from type III hypersensitivity. The mechanism by which this occurs appears to be multifactorial. Our working model is that infection suppresses FcγRI expression on eosinophils, which leads to decreased chemokine production and reduced cellular recruitment (Fig. 22). Because the immunological markers of type I hypersensitivity were decreased in chronically infected animals, *L. sigmodontis* may have the potential to protect against immediate hypersensitivity reactions under specific experimental conditions. Future studies would likely investigate the use of repeated infections to extend the length of infection, as this would allow for mast cell turnover to occur in an immune-regulated setting. Furthermore, future experiments using worm antigen preparations in place of live worm infection would allow for the characterization of the specific antigens responsible for host protection from hypersensitivity diseases.

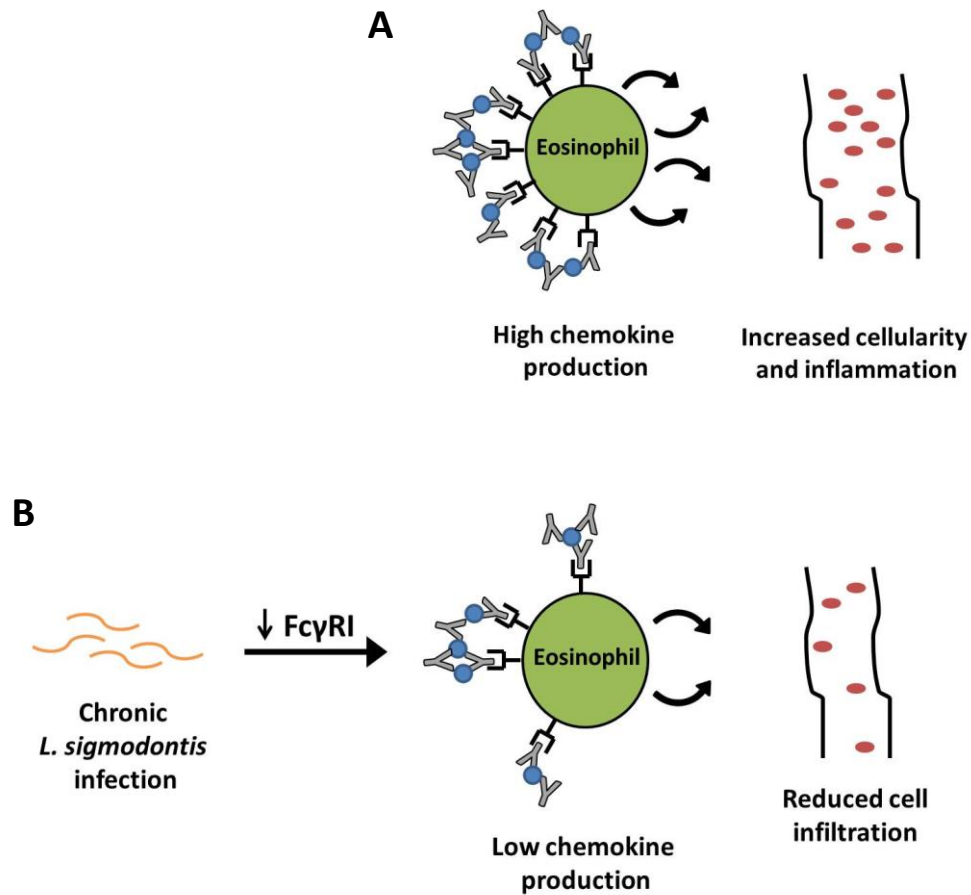


Figure 22. Working model for helminth protection from immune complex-mediated inflammation

(A) In sensitized animals, antibodies bind to OVA to form immune complexes. These complexes then ligate FcγRs on the surface of eosinophils to induce chemokine production. Neutrophils migrate to the tissue to induce inflammation and swelling. (B) In Sensitized + Infected animals, there is less FcγRI expression on eosinophils, therefore fewer receptors are ligated by immune complexes. Due to weaker stimulation, chemokine production is reduced, resulting in fewer neutrophils infiltrating to the site of immune complex deposition.

CHAPTER 4: Endothelial cells release soluble factors that support the *in vitro* survival of filarial worms

INTRODUCTION

Helminths are increasingly used as treatment options for allergy and autoimmune diseases. The administration of worm antigen is a highly attractive alternative to live infection; however it is difficult to obtain large numbers of worms and there can be variability among worm antigen preparations. The ability to perform immunologic experiments and characterize specific antigens that are therapeutic for allergy and autoimmunity is hindered by our inability to culture worms *in vitro*.

Additionally, the discovery of novel anthelmintics is hindered by our lack of an *in vitro* culture system that supports the survival of adult worms. The Global Program to Eliminate Lymphatic Filariasis (GPELF) is in need of short course macrofilaricides, yet a robust *in vitro* culture system is required before high throughput screening can commence. The development of an *in vitro* life cycle for filarial worms would provide 1) a mechanism for expanding filarial worms *in vitro* for the mass production of worm antigen and 2) a system for high throughput screening of drug candidates with macrofilaricide activity.

Before an *in vitro* life cycle can be developed, filarial worms need to survive *in vitro* for an extended period of time. To date, the majority of *in vitro* culture work has focused on worm molting (3; 129; 135; 162; 181), yet very little is known about the basic nutritional requirements of filarial worms. MF and L3 stage larvae lack a fully developed gut (19; 116), however MF are thought to utilize an “inner cell” food reserve during early development (122). In addition to the uptake of nutrients through the gut, all life cycle

stages have the ability to absorb low molecular weight nutrients directly through the cuticle (90; 208).

While co-culturing filarial worms with Rhesus monkey kidney cells (LLCMK₂) has been shown to induce molting (208), we sought to identify cell lines that could promote worm longevity *in vitro*. In this study, we found that a mouse endothelial cell line (EOMA) and a mosquito cell line (C6/36) prolonged the *in vitro* survival of microfilariae (MF) of *Litomosoides sigmodontis*. Culturing MF with EOMA cells in transwell plates also supported survival, indicating the cells secreted soluble factors that were beneficial to MF. We then performed a series of experiments to characterize the biochemical properties of the secreted factors. The factors are constitutively produced by EOMA cells as survival is also extended the use of conditioned media (CM), but only when the cells are cultured in the presence of serum. The factors are sensitive to heat treatment and lipid removal, and at least some are between 0.1-1 kDa in size. Finally, adult *L. sigmodontis* and *B. malayi* worms survive longer in EOMA CM than control media, indicating enhanced survival by endothelial cells is not stage or species-specific. The work represents the first steps toward the development of a chemically defined medium that provides filarial worms the nutrients required for long term *in vitro* survival.

RESULTS

Co-culturing MF with mouse endothelial cells extends *in vitro* survival from 5 days to 40 days

To characterize MF survival in basic cell culture media, we cultured MF in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS (Fig. 23A). We found that MF survive for a maximum of 5 days in this medium. Raising the FBS

concentration to 20% did not greatly extend survival, however lowering FBS to 0% expedited worm death.

When MF were co-cultured with a mouse endothelial cell line (EOMA) grown in DMEM with 10% FBS, survival was greatly enhanced (Fig. 23B). MF were able to survive for up to 40 days with no media exchange. Survival was also enhanced when MF were co-cultured with a mosquito cell line (C6/36) grown in Eagles Minimum Essential Medium (EMEM) supplemented with 10% FBS. Not all cell lines were capable of supporting MF survival, as MF cultured with a myeloma cell line only lived for 10 days.

Cells release soluble factors that are constitutively produced when cells are grown in the absence of FBS

To test whether MF required factors released by EOMA cells or direct contact with the cells to achieve prolonged survival, we cultured MF and EOMA cells in transwell plates. EOMA cells were cultured in the bottom reservoir and MF were cultured in the top reservoir, separated from the cells by a 0.22 μ m filter. Culturing MF in transwell plates with EOMA cells resulted in similar survival rates as co-culture, indicating that prolonged survival was due to soluble factors (Fig. 24A).

All stages of filarial worms release a complex excretory-secretory product that has been shown to have diverse effects on host cells. We next sought to determine whether MF were inducing EOMA cells to produce factors that necessary for survival, or if the cells were producing these factors constitutively. EOMA cells were cultured for 3 days in the absence of MF and the conditioned media (CM) was collected. When MF were cultured in CM survival was also greatly enhanced, with MF typically surviving for 20-25 days (Fig. 24B). This indicates that EOMA cells constitutively produce factors

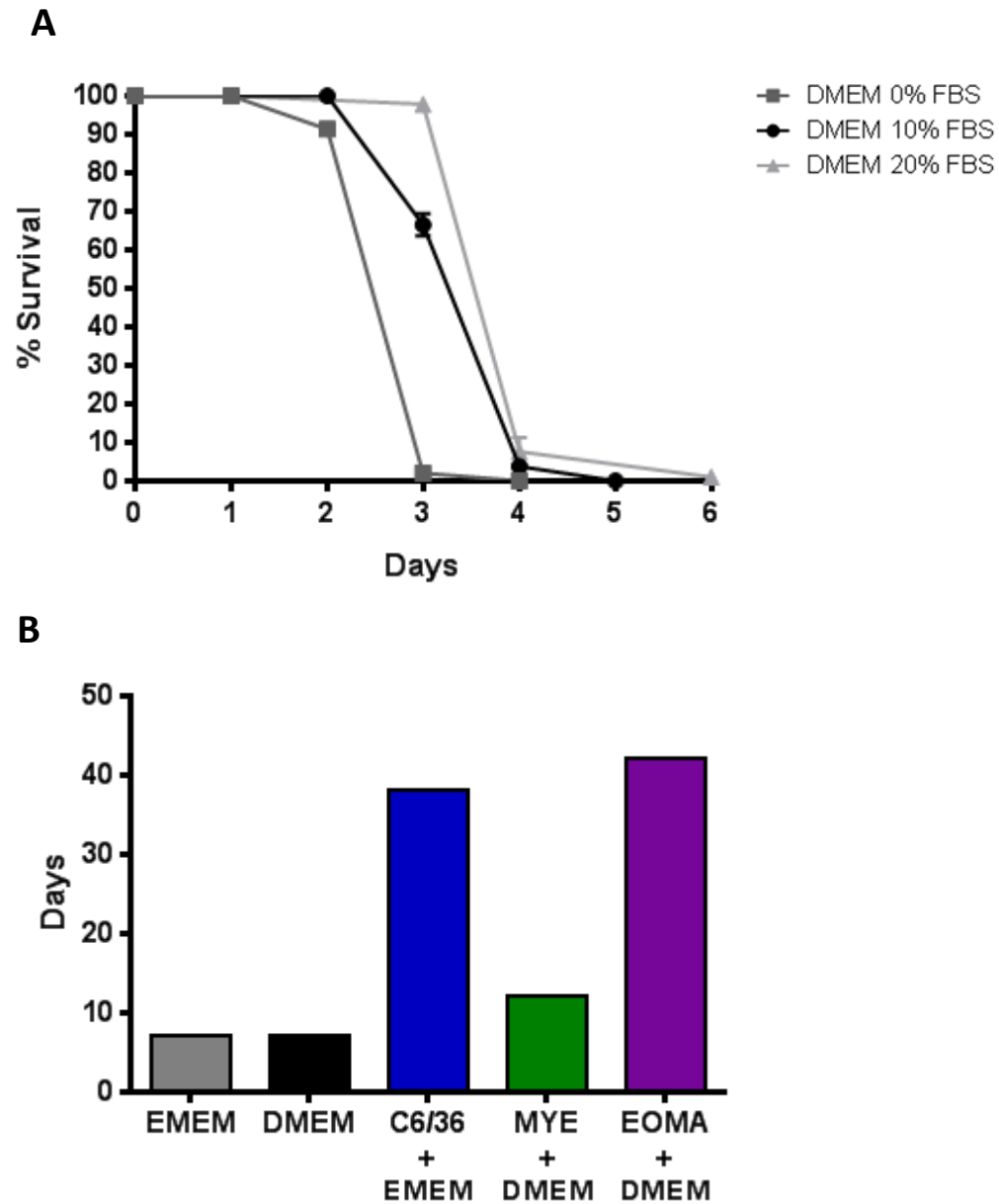


Figure 23. Culture of MF in basic cell culture media and with cell lines

Co-culture with mosquito and mouse endothelial cell lines enhances *in vitro* survival of MF. **(A)** Culture of MF in DMEM supplemented with increasing concentrations of FBS. **(B)** Co-culture of MF with mosquito (C6/36), mouse myeloma (MYE), and mouse endothelial (EOMA) cell lines.

that MF require for survival. Culturing MF in myeloma CM produced similar results to co-culture, with MF surviving for 8 days. Finally, culturing MF in CM from a rat basophilic leukemia cell line (RBL-2H3) did not extend survival beyond the media control (IMDM with 10% FBS).

FBS is a complex mixture of nutrients and can exhibit a high degree of batch-to-batch variability. In an effort to simplify culture conditions so that we may characterize the factors MF need to survive, we cultured EOMA cells in the absence of FBS (Fig. 24C). Interestingly, enhanced survival is not observed when MF are cultured in CM from cells grown in the absence of FBS (EOMA CM – FBS). Furthermore, the addition of 10% FBS to EOMA CM – FBS did not restore MF survival. This indicates that EOMA cells require FBS to produce the factors that are beneficial for MF survival.

Factors that support MF survival are between 0.1-1 kDa and are sensitive to heat treatment and lipid depletion.

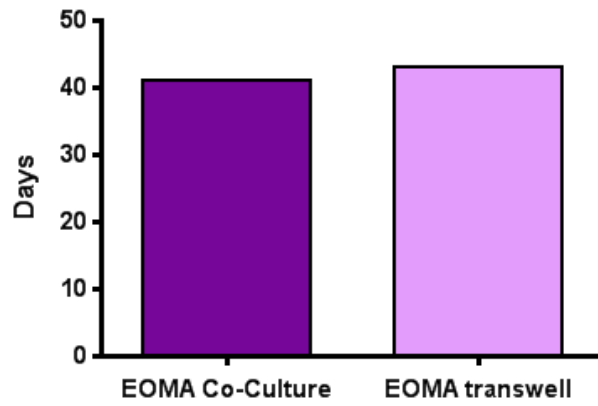
To elucidate the biochemical properties of the factors supporting MF survival, we performed heat treatment, lipid depletion, and dialysis on EOMA CM. Heat treating EOMA CM at 56°C for 1 hour resulted in a 50% reduction in worm survival (Fig. 25A). Heat treating the CM for a longer period of time (4 hours) did not further reduce survival. Importantly, MF survival was not affected by heat treating DMEM at 56°C. This indicates that EOMA CM contains heat labile factors which may potentially be proteins.

Heat treatment of EOMA CM at 100°C for 1 hour completely abrogated survival (Fig. 25B). The addition of 10% FBS to EOMA CM after 100°C heat treatment resulted in survival rates similar to DMEM with 10% FBS. MF survival in 100°C heat treated

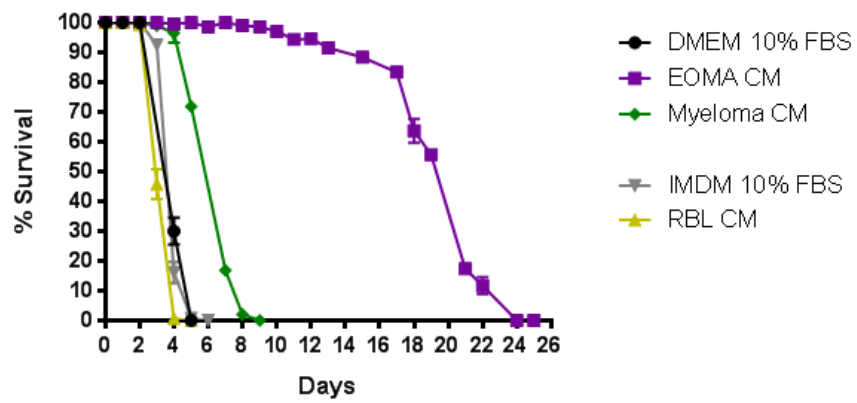
Figure 24. MF cultured in transwell plates, EOMA CM, and EOMA CM from cells grown in the absence of FBS.

Factors that prolong survival are soluble and constitutively produced when EOMA cells are grown in the presence of FBS. **(A)** Culture of MF in 0.22 μ m transwell plates. **(B)** MF cultured in conditioned media (CM). **(C)** Culture of MF in CM derived from cells grown for 3 days in the absence of FBS.

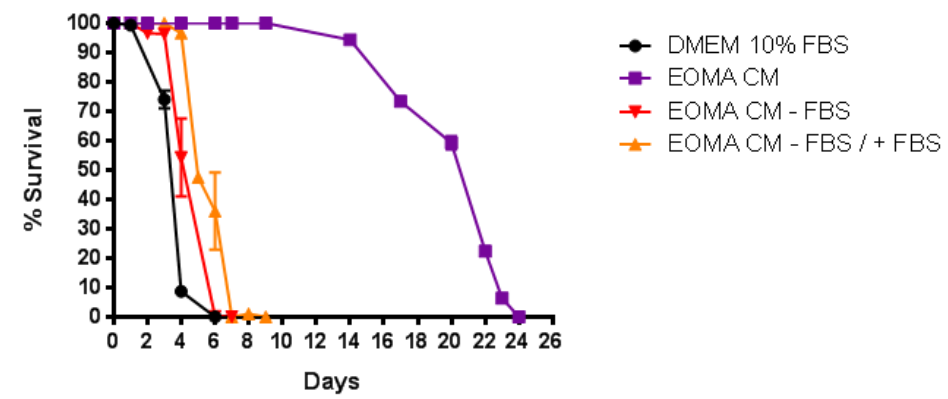
A



B



C



DMEM fell below the unheated DMEM control, regardless of whether or not 10% FBS was added back. These data indicate that EOMA CM also contains heat stable factors that MF require for survival.

We next used a commercially available lipid removal agent (LRA) to deplete EOMA CM of lipids and lipoproteins (Fig. 25C). Longevity of MF in lipid depleted EOMA CM was similar to the untreated DMEM control. This suggests the worms require a lipid factor released by EOMA cells. Because FBS contains lipids, we supplemented EOMA CM with 10% FBS after the LRA treatment, however it did not appreciably enhance survival. LRA treatment of DMEM resulted in similar survival curves as LRA treatment of EOMA CM.

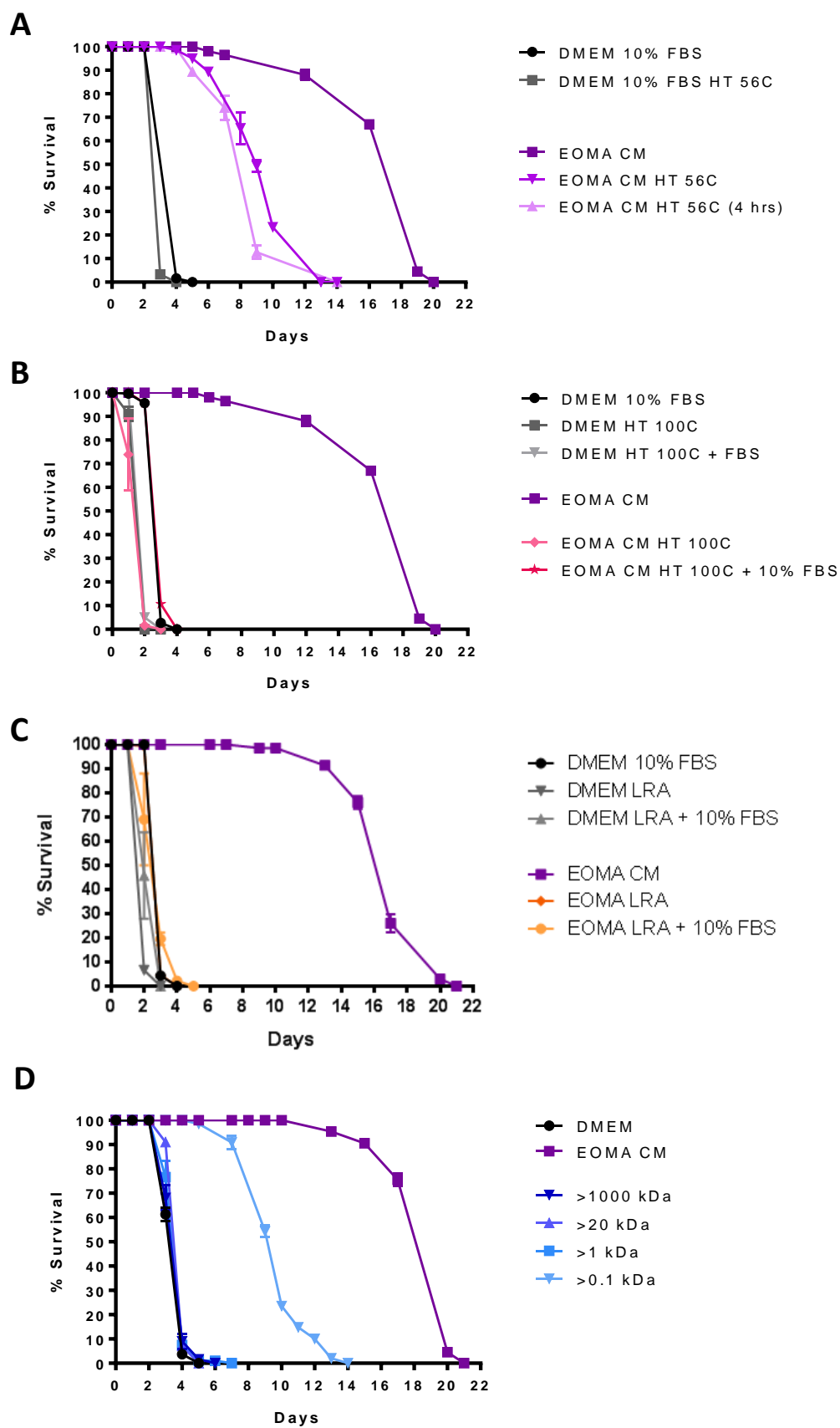
We performed dialysis to determine the size of the factors responsible for enhanced MF survival. EOMA CM was dialyzed into DMEM with 10% FBS using several molecular weight cutoffs (Fig. 25D). The only fraction that resulted in enhanced MF survival was the 0.1 kDa cutoff, indicating that the factors are larger than 0.1 kDa. Because the 1 kDa cutoff did not show enhanced survival, this indicates the factors are also smaller than 1 kDa. Therefore, the size of the factors falls between 0.1 and 1 kDa. Since none of the fractions completely restored MF survival to that of EOMA CM control, it is likely that multiple factors are acting in concert to promote MF longevity.

C3 is up-regulated in EOMA CM and enhances MF survival

Heat treatment of EOMA CM at 56°C reduced MF survival, suggesting that MF may require proteins present in EOMA CM. We ran a comparative 2D gel to determine

Figure 25. The effects of heat treatment, lipid removal, and dialysis on MF survival in EOMA CM

EOMA CM contains factors that are sensitive to heat treatment and lipid depletion, and are between 0.1-1 kDa in size. **(A)** DMEM and EOMA CM heat treated at 56°C for 1 or 4 hours. **(B)** DMEM and EOMA CM heat treated at 100°C for 1 hour. For some experiments 10% FBS was added after heat treatment. **(C)** DMEM and EOMA CM were depleted of lipids and lipoproteins with a lipid removal agent (LRA). For some experiments 10% FBS was added after lipid removal. **(D)** MF culture in EOMA CM dialyzed into DMEM with 10% FBS using 1000, 20, 1, and 0.1kDa molecular weight cutoffs.



which proteins are up-regulated in EOMA CM compared to RBL-2H3 CM, which does not support MF survival. There were 32 unique spots that were up-regulated in EOMA CM (Fig. 26), which we then identified by mass spectrometry (Table 1). A cluster analysis of the up-regulated proteins resulted in four functional groups: regulation of cell death, secreted/extracellular, nucleotide binding, and metal ion binding (Table 2).

Due to the soluble nature of the factors that support MF survival, we were interested in supplementing DMEM with 4 candidate proteins from the secreted / extracellular functional group: albumin, apolipoprotein E, C3, and collagen. The addition of a single dose of 20 µg of recombinant mouse C3 slightly extended the *in vitro* survival of MF, whereas recombinant apolipoprotein E and purified albumin did not prolong survival (Fig. 27). The addition of purified collagen was toxic to the MF (data not shown), potentially due to the buffer collagen was stored in.

Lipid and nucleoside supplements do not prolong MF survival

In previous studies, mammalian-derived serum was the only lipid-rich cell culture supplement available for culturing filarial worms *in vitro* (208). With chemically defined serum replacements now commercially available, we sought to determine whether they could support MF survival. Cell-Ess® is a synthetic serum replacement that contains lipids and growth factors for *in vitro* cell culture. Despite supporting the growth of primary cells and cell lines, DMEM supplemented with 10% Cell-Ess® was toxic to MF (Fig. 28A). Similarly, when DMEM was supplemented with a Fatty Acid Supplement (Sigma) MF died before the DMEM with 10% FBS control (Fig. 28A).

Filarial worms have previously been shown to lack pathways for *de novo* synthesis of sterols (65). Although FBS contains cholesterol, we wanted to determine if

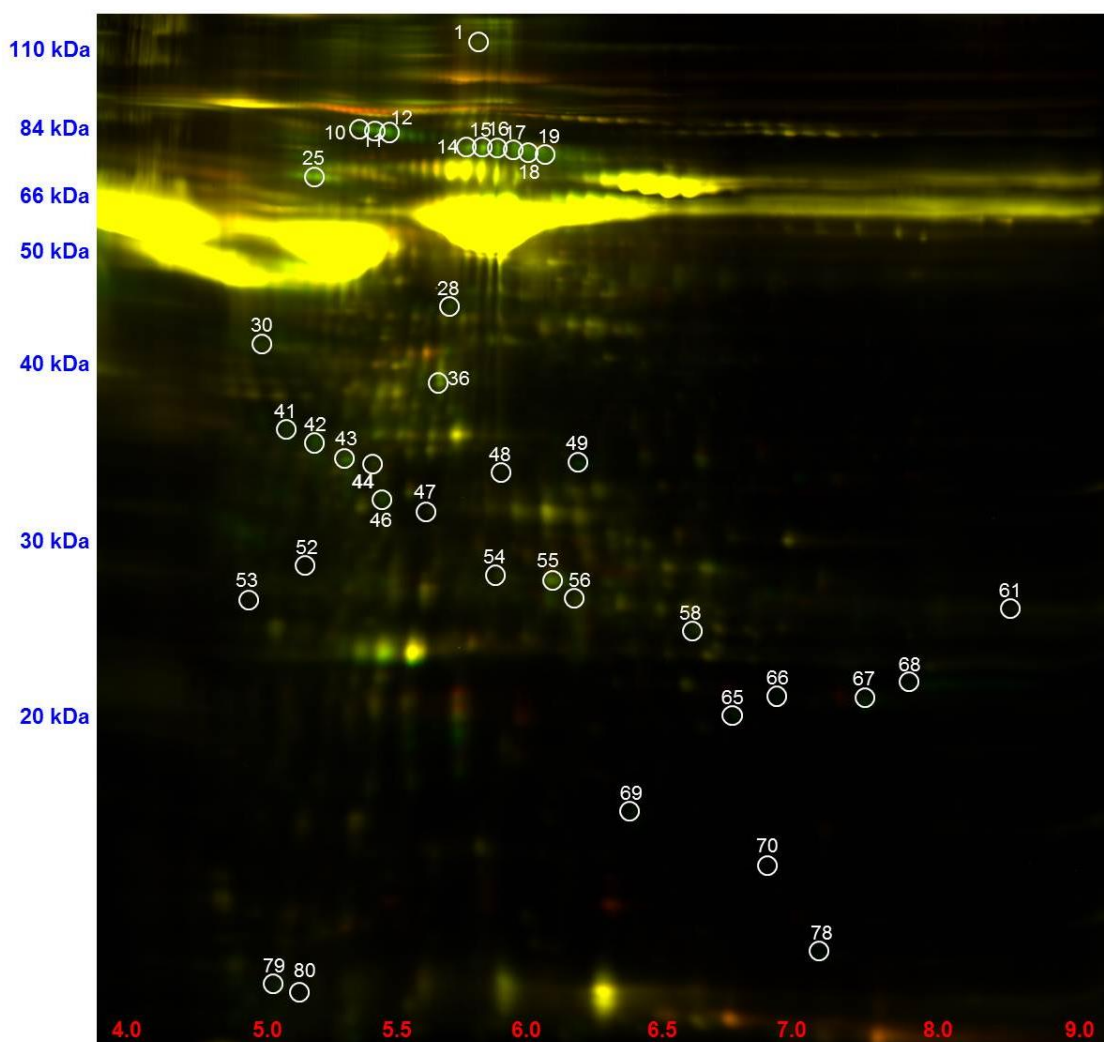


Figure 26. Comparative 2D-DIGE of EOMA CM and RBL-2H3 CM

Comparative gel of EOMA CM (green) and RBL-2H3 CM (red). 32 spots were identified as upregulated > 1.5 fold in EOMA CM compared to RBL-2H3 CM.

Spot number	Match Confidence	Protein Name [Species]	Accession No.	Protein MW	Protein PI
1	High	Serum albumin	ALBU	68,648	5.8
11	Low	Alstrom syndrome protein 1 homolog	ALMS1	359,993	6.0
15	High	Serum albumin	ALBU	68,648	5.8
18	High	Voltage-dependent R-type calcium channel subunit alpha-1E	CAC1E	257,071	8.5
25	High	Nucleolar RNA helicase 2	DDX21	93,493	9.2
28	High	Serum albumin	ALBU	68,648	5.8
30	High	Complement C4 [Bos taurus]	gi 31563307	101,820	6.2
36	High	Complement C3	CO3	186,366	6.3
41	High	Keratin, type II cytoskeletal 6B	K2C6B	60,285	8.5
42	High	Ectodysplasin-A receptor-associated adapter protein	EDAD	23,738	5.0
43	High	Ectodysplasin-A receptor-associated adapter protein	EDAD	23,738	5.0
44	High	Apolipoprotein E	APOE	35,844	5.6
46	High	Collagen alpha-1(I) chain	CO1A1	137,948	5.7
47	High	Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein) [Bos taurus]	gi 59857769	101,446	6.3
48	High	Serum albumin	ALBU	68,648	5.8
49	High	Cathepsin L1	CATL1	37,523	6.4
52	Low	Transcription factor EC	TFEC	35,122	6.3
53	Low	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADV	70,831	8.9
54	High	Serum albumin	ALBU	68,648	5.8
55	High	Complement C3	CO3	186,366	6.3
56	High	Complement C3	CO3	186,366	6.3
58	High	Serum albumin	ALBU	68,648	5.8
61	High	Proteasome subunit alpha type-7	PSA7	27,838	8.6
65	High	Lymphoid-restricted membrane protein	LRMP	59,551	5.1
66	High	Peroxisomal protein 1	PRDX1	22,162	8.3
67	High	Centrosomal protein C10orf90 homolog	CJ090	71,476	8.8
68	High	C2 calcium-dependent domain-containing protein 4D	C2C4D	36,842	11.0
69	Low	Tripartite motif-containing protein 43B	TR43B	52,204	8.6
70	High	Nucleoside diphosphate kinase B	NDKB	17,352	7.0
78	High	Histone H2A.Z	H2AZ	13,545	10.6
79	High	E3 ubiquitin-protein ligase RNF181	RN181	19,088	5.7
80	Low	Replication factor C subunit 5	RFC5	38,072	7.7

Table 1. Mass spectrometry identification of spots upregulated in EOMA CM

Identification of 32 upregulated spots from 2D DIGE gel (Fig. 26). All proteins were of mouse origin, except for spot 30 (C4) and spot 47 (inter-alpha globulin inhibitor H4) which originated from *Bos taurus*.

Functional Group	Enrichment Score	Genes	Accession No.
Regulation of cell death	1.59	Peroxiredoxin-1	PRDX1
		Nucleoside diphosphate kinase B	NDKB
Secreted / extracellular	0.82	Albumin	ALBU
		Apolipoprotein E	APOE
		Complement C3	CO3
		Collagen alpha-1 chain	CO1A1
Nucleotide binding	0.47	Nucleolar RNA helicase 2	DDX21
		Very long-chain specific acyl-CoA dehydrogenase (mitochondrial)	ACADV
		Nucleoside diphosphate kinase B	NDKB
		Replication factor C subunit 5	RFC5
Metal ion binding	0.05	Albumin	ALBU
		Voltage-dependent R-type calcium channel subunit alpha-1E	CAC1E
		Nucleoside diphosphate kinase B	NDKB
		Replication factor C subunit 5	RFC5

Table 2. Cluster analysis of proteins upregulated in EOMA CM.

The overall enrichment score for the group is based on the scores of each term members.

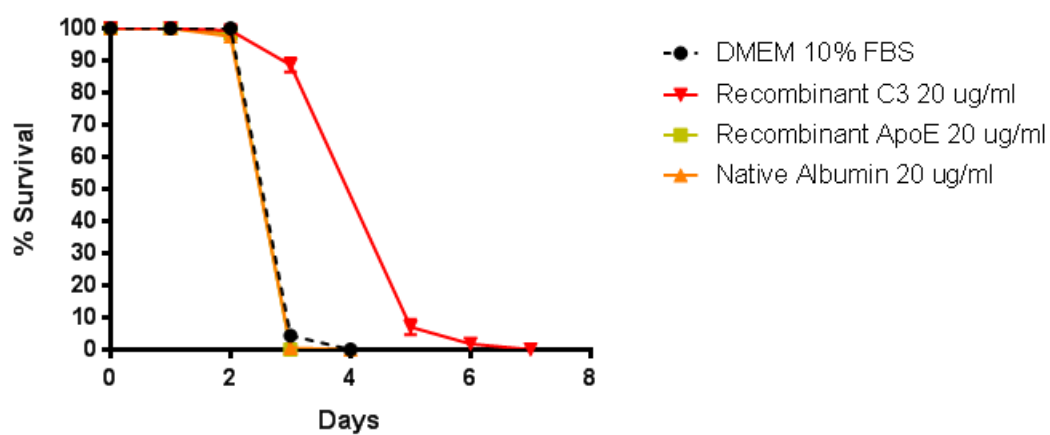


Figure 27. Supplementation of DMEM with proteins upregulated in EOMA CM

A single dose of 20 μ g of recombinant mouse C3, apolipoprotein E, or native albumin was added to DMEM with 10% FBS. C3 slightly prolongs MF survival.

the addition of extra cholesterol would be beneficial for the MF. We added 10 µg/ml of cholesterol to DMEM with 10%FBS, but found it had no effect on MF survival (Fig. 28A). Finally, because filarial worms also lack pathways for *de novo* purine synthesis, and because nucleotide binding proteins were a functional group that was upregulated in EOMA CM, we added a nucleoside supplement to DMEM with 10% FBS (Fig. 28B), however the addition of exogenous nucleosides also had no effect on worm survival.

EOMA CM prolongs adult worm survival for *L. sigmodontis* and *B. malayi*

To determine whether the factors that prolong MF survival could support other developmental stages, we cultured *L. sigmodontis* adult female worms with EOMA cells in transwell plates. With no media exchange, adult *L. sigmodontis* worms survived longer in the presence of EOMA cells (Fig. 29A), indicating that the beneficial factors do not act on specific larval stages. Culturing 1 worm in 1 ml of CM, exchanged every other day to accommodate the high metabolic rate of adult female worms, resulted in similar survival profiles as MF (Fig. 29B). EOMA CM provided the best results, however increased survival was lost when worms were cultured in EOMA CM – FBS. RBL CM did not increase survival beyond the DMEM with 10% FBS control. Finally, heat treatment at 100°C dropped adult survival below DMEM, indicating it had a toxic effect.

The advent of an *in vitro* culture system for filarial worms that infect humans, such as *B. malayi*, would assist in the search for novel macrofilaricides as it would allow for high-throughput screening of candidate drugs. We cultured *B. malayi* adult female worms in CM to test whether the factors that promoted survival were species-specific. Again, EOMA CM provided the best results, with worms surviving as long as 66 days *in vitro* with media exchanged every other day (Fig. 29C). DMEM with 10% FBS also

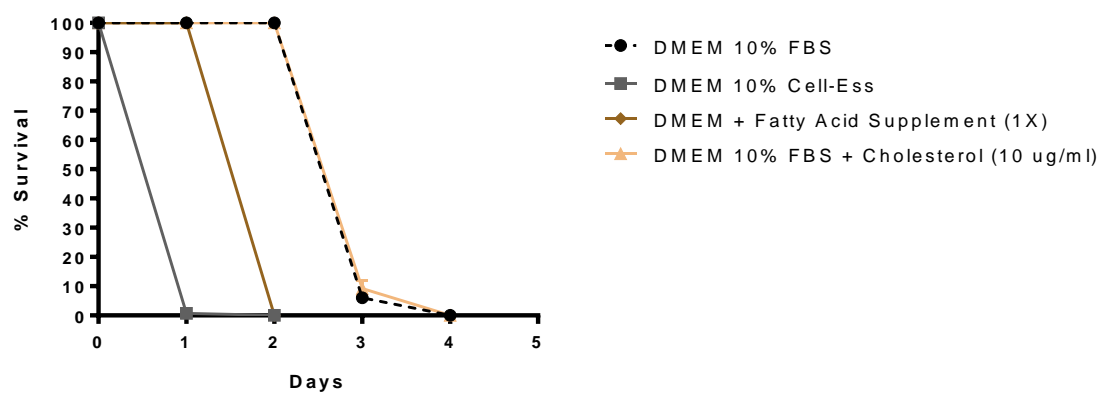
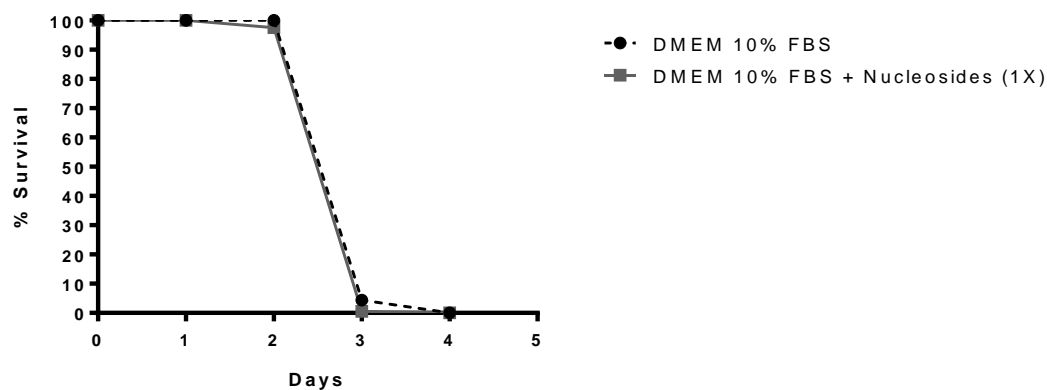
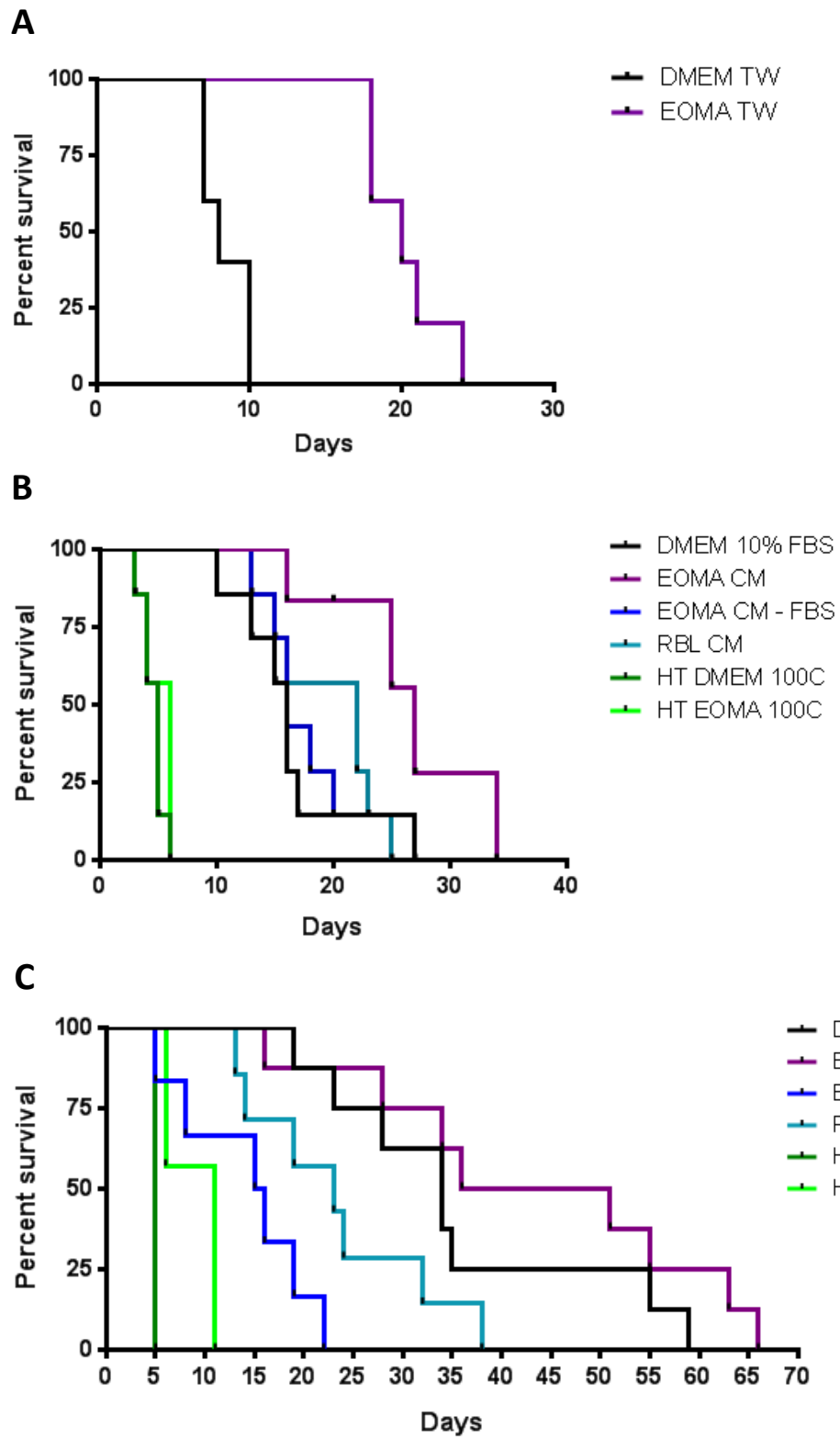
A**B**

Figure 28. MF cultured with serum replacements, cholesterol, and nucleoside supplements.

Use of serum replacements, added cholesterol or nucleosides did not enhance MF survival. **(A)** DMEM supplemented with 10% Cell-Ess or 1X Fatty Acid Supplement as serum replacements. 10 μ g/ml of cholesterol was also added to DMEM with 10% FBS. **(B)** A nucleoside supplement (1X) was added to DMEM with 10% FBS.

Figure 29. *In vitro* survival of adult filarial worms

EOMA CM prolongs adult worm survival of *L. sigmodontis* and *B. malayi*. **(A)** Culture of *L. sigmodontis* adult female worms in 0.22 transwell plates with EOMA cells. **(B)** *L. sigmodontis* adult female worms cultured in CM. One adult female worm was cultured per 1 ml of CM, and CM was exchanged every other day. **(C)** *B. malayi* adult female worms were cultured in CM under similar experimental conditions.



resulted in enhanced longevity, albeit it was slightly less than EOMA CM. Heat treatment was detrimental to worm survival, and prolonged survival was lost when EOMA cells were grown in the absence of FBS. Overall, survival curves for the *in vitro* culture of filarial worms show similar trends, regardless of the stage or species of parasite used.

DISCUSSION

In this study, we have shown that mouse endothelial cells (EOMA) and mosquito cells (C6/36) support the *in vitro* survival of *L. sigmodontis* microfilariae. Although co-culturing cells with MF results in the longest survival time, culturing MF in transwell plates or EOMA CM provides comparable results, indicating the cells produce soluble factors required for worm survival. EOMA cells require FBS to produce these factors, and the factors are sensitive to heat treatment and lipid removal. Dialysis experiments show that factors between 0.1 – 1 kDa are responsible for roughly half of the extended survival. Importantly, EOMA CM also supports the survival of both *L. sigmodontis* and *B. malayi* adult worms.

Although the *B. malayi* genome has been sequenced (70), a large portion is comprised of unique or hypothetical proteins. KEGG pathway analysis reveals that filarial worms lack many proteins involved in important pathways, including heme, riboflavin, and purine biosynthesis (44). This presents two possibilities: 1) filarial worms have evolved unique enzymes for the synthesis of essential nutrients, or 2) the worms acquire necessary precursors or end products from the host. *Wolbachia* have been hypothesized to contribute to worm survival because they utilize biosynthesis pathways to produce heme, riboflavin and purines. However, because *Loa loa* lacks both *Wolbachia* as well as the aforementioned pathways, the contribution of *Wolbachia*-

derived nutrients is likely minimal, with worms primarily relying on the host for these factors (44).

Unsupplemented DMEM contains important salts, metals, amino acids, and vitamins (including riboflavin). Heat treatment of riboflavin at 100°C for 1 hour has been shown to reduce the quantity of active vitamin by 50% (56), and may account for the decreased survival observed with heat treated DMEM. FBS contains trace amounts of hemoglobin and therefore may be an important source of heme (229). Denaturation of hemoglobin is reversible at low temperatures but irreversible at 100°C (147), which may further explain why heating DMEM to high temperatures is toxic to MF (Fig. 25B).

Although filarial worms lack purine biosynthesis pathways, there is evidence they can convert pyrimidines to purines (44). Our data demonstrate that the addition of exogenous nucleosides does not enhance survival (Fig. 28B). However, MF may respond better to exogenous purine precursors or the addition of a carrier protein, as EOMA CM was enriched for nucleotide binding proteins compared to RBL-2H3 CM (Table 2).

Helminths also lack pathways for *de novo* synthesis of cholesterol. *W. bancrofti* (185) and *S. mansoni* (33) have LDL receptors, indicating that these parasites may acquire cholesterol and other sterols from the host. In fact, adult schistosome worms contain cholesterol while cercariae contain sterols derived from the intermediate snail host (66). Helminth infections have also been shown to alter the lipid profiles of infected patients (16), though whether this is related to worm survival or disease pathogenesis remains unclear.

FBS contains cholesterol as well as lipid carriers HDL, LDL, VLDL, and chylomicrons. Lipid carriers are stable when subjected to low temperatures (56°C) and

denature at high temperatures (100°C) (98; 132); therefore, they would not be affected by 56°C heat inactivation of FBS. Because both culturing MF in DMEM with 10% FBS and supplementing with extra cholesterol does not promote long-term survival, cholesterol may be an important nutrient but play a minimal role in determining MF longevity.

EOMA cells grown in the absence of FBS (Fig. 24C) and chemically defined FBS replacements (Fig. 28A) fail to support MF survival. Although EOMA cells may be using FBS to synthesize and release factors, there is a possibility that the cells may simply be altering components present in FBS into derivatives that are more useful for the worms. For example, EOMA cells express lipoprotein lipase (LPL) on the cell surface which can release triglycerides from very low density lipoprotein (VLDL) and chylomicrons present in FBS. Therefore, instead of directly producing nutrients MF require, EOMA cells may catalyze the release of sequestered nutrients present in FBS.

Dialysis experiments indicate that factors between 0.1 and 1 kDa in size are responsible for half of the prolonged survival observed with EOMA CM (Fig. 25D). Potential factors in the 0.1 – 1 kDa size range include vitamins, nucleotides, amino acids, monosaccharides, fatty acids, cholesterol, porphyrins (including heme), and lipopeptides. These small molecules are the building blocks for macromolecules, and the direct uptake of these factors from the host may explain the lack of biosynthesis pathways present in filarial worms. The small size of the beneficial factors is supported by previous observations that MF absorb low molecular weight substances directly through the cuticle (90).

Given that none of the fractions resulted in complete restoration of MF survival similar to EOMA CM (i.e. survival for 20-25 days), it is possible that necessary factors

are also present in the larger fractions and that they are carrier proteins. Because small molecules would not be retained by the larger cutoffs, empty carrier proteins would lose their ability to prolong MF survival because their cargo is absent. Due to the heat and LRA-sensitive nature of EOMA CM, it likely contains both protein and lipid components that are beneficial for MF survival.

In addition to being a nutrient, factors in EOMA CM may provide an environmental cue or signal that promotes survival. Nematodes have cuticular sensilla that allow the parasite sense its location during its complex life cycle (13). Neurons present in the sensilla can receive chemosensory, mechanosensory, and thermosensory signals (13).

We found that the addition of recombinant mouse C3 slightly prolongs MF survival (Fig. 27). The likelihood that C3 is an essential nutrient is low, as survival was only extended by a couple days. Therefore, it is possible that the C3 present in EOMA CM provides a chemosensory signal to indicate MF are located in the mammalian host. Future studies would need to administer repeat doses of C3 to see if survival can be further prolonged, as well as determine whether C3a or C3b is the important component. There are no C3a or C3b receptors in the *Brugia malayi* genome that are homologous to human complement receptors. The identification of filaria-specific complement receptors may provide useful targets for vaccines or rational drug design.

An in-depth understanding of the nutrients and environmental cues filarial worms need to survive is an important step toward the development of novel anthelmintics. The GPELF would benefit greatly from effective short-course macrofilaricides, yet drug discovery is hindered by our inability to culture adult filarial worms. We have

demonstrated that culturing both rodent and human filarial worms in EOMA CM extends *in vitro* survival (Fig. 29). By prolonging the survival of filarial worms, investigators can employ high throughput screening to test candidate pharmaceuticals.

Prolonging *in vitro* survival is also the first step toward the development of an *in vitro* life cycle, which would shed light on the biology of larval development and potential lead to the identification of additional drug targets. Furthermore, worm recovery from animal hosts is often low, limiting the types of experiments that can be performed due to a lack of worm antigen and excretory-secretory product. An *in vitro* life cycle would bypass this problem by allowing for the expansion of filarial worms *in vitro* and mass production of worm antigen with high consistency. This is crucial for research involving the use of helminths to treat allergy and autoimmune diseases. Because filarial worms cause host pathology and disease, the characterization and administration of protective worm antigens is a highly desirable alternative to live infection.

A comparison of the nutrients, growth factors, and lipids present in FBS versus FBS replacements may provide insight into which classes of molecules are essential for worm survival. Due to high variability of FBS among lots, the ultimate goal would be to extend the *in vitro* survival of filarial worms in serum-free culture media. Future studies would also employ the use of mass spectrometry to chemically define factors present in the 0.1 – 1 kDa fraction of EOMA CM but absent in RBL-2H3 CM. Supplementing DMEM with the identified factors, and potentially carrier proteins, would be the next step toward the development of a culture media designed to support filarial worm survival.

CHAPTER 5: Discussion

PREFACE AND SIGNIFICANCE

Humans have been infected with parasitic helminths for thousands of years. In the effort to improve health through the elimination of infectious diseases, the beneficial aspects of helminth infection may have been overlooked. Although capable of causing severe morbidity and mortality, many individuals harbor asymptomatic infections. Furthermore, there have been numerous studies showing an inverse correlation between helminth infection and immune system disorders. Allergic and autoimmune disorders are thought to arise from a lack of established immunoregulatory networks, which are a hallmark of chronic helminth infection. This highlights the potential for helminths to serve as a tool for developing new therapies to combat diseases associated with hyperresponsive immune systems.

The mechanisms by which helminths regulate immune responses are still poorly understood. Immunoregulatory networks enable prolonged survival of the parasites and minimize host pathology. Although the host initially develops a Th2 response to combat infection, helminths subsequently modulate both Th2 and Th1 arms of the immune system (14; 144). This primarily occurs by the differentiation of FoxP3⁺ regulatory T cells and the production of immunosuppressive cytokines, such as IL-10 and TGF- β (78). In addition to extrinsic regulation by regulatory T cells, Th2 effector cells can enter a state of exhaustion or anergy due to chronic immune activation (222). T cell exhaustion is exemplified by sustained expression of inhibitory receptors, poor effector function, and an altered transcriptional state compared to functional T cell. T cells are described as anergic when they are inactivated after previous stimulation, which can occur due to an absence of co-stimulatory signals or adaptive tolerance. Specific worm antigens and

secreted products have also been shown to directly suppress immune cells (76; 82; 175). Further regulation of the immune system is achieved by helminth expression of orthologs for host cytokines (75; 228).

Studies have found that helminth infection can prevent the development of allergic disease, however very few have evaluated the effects of infection when administered after allergic sensitization (55). This body of work provides an in-depth investigation of the therapeutic potential of helminth infection for pre-existing allergic disease. It is also the first study to evaluate the effect helminth infection has on 1) mast cells and basophils in the context of allergen-induced type I hypersensitivity, 2) protection against immune complex-mediated inflammation, and 3) glomerular damage caused by systemic lupus erythematosus. In addition, we provide a foundation for the *in vitro* culture of filarial worms so that specific worm antigens, in lieu of live infection, may be used as therapeutics for diseases caused by dysregulation of the immune system.

SPECIFIC AIM 1

Ten weeks of *L. sigmodontis* infection did not protect against clinical type I hypersensitivity, which may indicate that helminths are better at preventing allergic disease than treating it. However, a longer lasting infection may further decrease immunological markers of type I hypersensitivity, such as basophil and mast cell activation, to a threshold that corresponds to clinical protection. Basophils from infected mice have been shown to be hyporesponsive to IgE cross-linking in an IL-10 dependent manner (114). This work expands on these findings to show that basophils are also suppressed in response to allergen stimulation.

Infection did provide clinical protection against type III hypersensitivity in a cutaneous ear challenge model. Protection appears to be mediated by reduced Fc γ RI expression on eosinophils. Eosinophils are important for worm clearance (111), and previous research demonstrates that worms and worm products can suppress eosinophil function (168; 193; 202).

Our work also supports previously published data showing that helminth infection reduces levels of circulating antigen-specific antibodies (17; 47; 127; 164; 167; 180; 231). We show that the reduction in allergen-specific antibodies also occurs when infection is given after sensitization, and that infection can reduce autoantibodies in mice with SLE. Furthermore, infection suppresses mesangial cell proliferation 24 weeks after administration of pristane. These results are in agreement with studies that have shown helminth infection and worm products are capable of suppressing cell proliferation (7; 8; 82)

Limitations

Though we have demonstrated an association between reduced neutrophil-specific chemokine production by eosinophils and decreased Fc γ RI expression, we have not yet performed experiments to test whether there is a causal relationship between eosinophil function and immune complex-mediated ear swelling. Ideally, the transfer of eosinophils from Sensitized + Infected animals to eosinophil-deficient Sensitized animals would allow us to conclude that eosinophils are the primary cell type responsible for host protection from immune complex-mediated inflammation. In order to obtain enough eosinophils for adoptive transfer, we would need to use IL-5 transgenic (IL-5tg) mice (121). A potential setback to this approach is that, although available on a BALB/c

background, IL-5tg mice display enhanced resistance to *L. sigmodontis* infection in an eosinophil-dependent manner (141). Thus, clearance of worms may prevent *L. sigmodontis*-induced suppression of eosinophils in IL-5tg mice.

Significant barriers also exist with respect to obtaining eosinophil-deficient mice. A monoclonal antibody that targets the eotaxin receptor CCR3 has been shown to deplete eosinophils *in vivo* (79). However, this reagent is not currently commercially available. Eosinophil-deficient mouse strains PHIL (119) and Δ dblGATA (92) have been criticized for having non-specific effects on immune cells, such as impaired basophil function (161). A recently developed Cre-Lox system for the selective depletion of eosinophils (48) may potentially be used for transfer experiments once animals are backcrossed onto a BALB/c background.

Another limitation to this study is that we have not identified a mechanism to explain the reduced numbers of macrophages present in the ear 24 hours after OVA challenge. We did observe that infection slightly decreased levels of multiple monocyte-specific chemokines; however, an alternative explanation is that the strong Th2 response induced by infection may be affecting local macrophage proliferation rather than monocyte recruitment from the blood (99). Baseline numbers of resident macrophages in the ear were not enumerated prior to OVA challenge, and the only time point assayed for chemokine production in the ear was 6 hours post-challenge. Thus, we are currently unable to determine whether monocyte specific chemokines are significantly downregulated at other time points, or whether infection is preventing local macrophage proliferation in the ear.

Future directions

Ten weeks of *L. sigmodontis* infection reduced immunological markers of type I hypersensitivity; therefore, infection may have the potential to protect against clinical disease if infection occurs for a longer period of time. For instance, assessment of local anaphylaxis at 12 weeks post-infection or after repeat infections may show protection against clinical disease. Repeat or prolonged infections would allow for the turnover of mast cells and would provide the best possible scenario to observe whether protection is feasible. These experiments will help determine if helminth infections can treat type I hypersensitivity, or if they are better suited for preventing the development of allergic disease.

The observation that chronic infection was associated with reduced Fc γ RI expression raises the possibility that other receptors might also be affected. In addition to Fc γ RI, Fc γ RII, and Fc γ RIII, mice also express Fc γ RIV. Fc γ RI, Fc γ RIII, and Fc γ RIV are activating receptors whereas Fc γ RII is an inhibitory receptor. Fc γ RIII and Fc γ RIV are thought to have redundant roles (95), however we did not specifically evaluate the role of Fc γ RIV in our cutaneous model of type III hypersensitivity.

Future experiments would also explore potential differences in complement receptor expression. Because we show slightly increased complement participation in immune complexes from Sensitized + Infected animals, and previous studies have implicated complement binding with enhanced immune complex clearance (43; 97; 107), changes in complement receptor CR1 expression may highlight an additional mechanism of immune suppression.

Other studies have emphasized the importance of C5 in the pathology of reverse Arthus reactions (18; 88). We would like to monitor C5a production following OVA

challenge, the presence of C5 in immune complex formation via immunohistochemistry, and changes in C5a receptor expression to determine if C5 is important in our allergy model. C5/C5a levels were slightly reduced in the ears of Sensitized + Infected animals 6 hours after OVA challenge (Fig. 18A). Performing the chemokine array on ear tissue 1 or 2 hours after challenge may show enhanced differences between groups with regards to complement activation.

We would also like to determine whether there are changes in immune complex composition in our SLE model. Using immunohistochemistry to stain kidney sections for IgG and complement will allow us to assess the size of immune complexes as well as the role of complement in the setting of SLE. We hypothesize that because infection decreased anti-dsDNA autoantibodies, we will observe immune complexes with a similar phenotype to those found in Sensitized + Infected animals. More specifically, Pristane + Infected animals will form complexes that are larger in size and have increased complement activation.

Our data suggest that antibody production is not impaired during the course of infection, leading us to hypothesize that antibody catabolism may be increased during chronic *L. sigmodontis* infection. We speculate that antibodies produced in response to infection increases host antibody catabolism in an effort to maintain homeostasis. This increased catabolism would result in lower levels of antigen-specific antibodies in the presence of high levels of total antibody. To test this, we plan to sensitize and infect FcRn knockout mice and monitor antibody levels throughout the course of infection. FcRn knockout mice lack the neonatal Fc gamma receptor which dictates the half-life of circulating IgG (71). If infection is affecting antibody catabolism by inducing high levels

of total IgG, we would expect that helminth infection would fail to reduce allergen-specific antibody levels in FcRn knockout mice. Additionally, we can investigate changes in antibody catabolism by injecting non-specific antibodies into naïve and chronically infected animals and monitor the rate of antibody clearance over time.

Finally, we would like future studies to focus on elucidating the mechanism by which infection decreases FcγRI expression on eosinophils. Administering repeat injections of LsAg to previously sensitized mice will determine if changes in FcγRI expression can occur in the absence of live infection. The next step would include culturing eosinophils *ex vivo* with *L. sigmodontis* antigen or excretory-secretory product. If decreased FcγRI is observed in this setting, we can conclude that worm products act directly on eosinophils to alter expression of surface Fc gamma receptors. In addition to measuring Fc gamma receptors, expression of cell signaling molecules, such as Syk and Akt, can be assessed following *in vitro* stimulation with LsAg. Both Syk and Akt have previously been shown to be downregulated in basophils during chronic infection (114), and may provide an alternative explanation for reduced cytokine production by eosinophils. Antigens can then be fractionated and analyzed to determine which components are responsible for eosinophil suppression. The identification of specific worm antigens responsible for eosinophil suppression could lead to the development of novel therapeutics for eosinophil-mediated diseases.

SPECIFIC AIM 2

Little is known about the nutritional requirements of filarial worms. Filarial worms lack pathways for *de novo* synthesis of purines, heme, riboflavin, and sterols (44; 65), indicating they may acquire these nutrients from the host. Previous research has

demonstrated that co-culturing filarial worms with mammalian cells is beneficial for molting (208). Our studies indicate that co-culture can also significantly prolong *in vitro* survival.

EOMA cells release soluble factors that are constitutively produced in the presence of FBS. This aspect allows for the mass production and long term storage of EOMA CM, reducing variability among experiments and simplifying the culture system to a cell-free state. Cell culture supernatants are easy to obtain and are readily available for biochemical analysis. Based on the preliminary work in this study, we are poised to identify specific nutrients MF and adult filarial worms require for long term *in vitro* survival. Designing a chemically defined culture media that supports the survival of all larval stages is the first step toward the development of a self-sustaining *in vitro* life cycle. The results of our studies to date demonstrate that filarial worms require nutrients that have a lipid moiety and are heat labile. In addition, worms exhibit prolonged survival when cultured with factors from EOMA CM that are between 0.1 and 1 kDa in size. Potential micronutrients that may be beneficial for worm survival include lipopeptides, sterols, vitamins, and porphyrins.

Limitations

One of the major limitations of this study is the requirement of serum for enhanced worm survival in EOMA CM. FBS is a complex mixture of nutrients that can vary considerably among lots, making it inherently difficult to analyze factors produced by endothelial cells in the presence of FBS. While the 2D gel revealed 32 proteins upregulated in EOMA CM, we undoubtedly missed proteins that were present at low concentrations. In an attempt to remedy this, we tried culturing MF in commercially

available serum replacements. Surprisingly, not only did these replacements not support survival, but they tended to be toxic to filarial worms.

We observed that heat treatment at 56°C reduced survival by 50%, indicating that proteins likely play a role in enhanced survival. However, the hypothesis that MF require proteins could not be confirmed by protease digestion of EOMA CM. Typical digestion methodology involves the inactivation of proteases by heat treating the sample at 95°C for 10 minutes. We found that this did not result in 100% inactivation of proteases, as MF were killed when cultured in protease-treated media. Additionally, the use of chemical protease inhibitors also proved to be toxic to MF.

A synthetic calcium silicate hydrate lipid removal agent (LRA) was used to deplete lipids from EOMA CM. The non-specific removal of all molecules containing lipid moieties prevents us from distinguishing whether MF are utilizing lipids, lipopeptides or lipoproteins present in EOMA CM. Also, LRA has also been shown to remove genomic DNA (240). Although we do not anticipate genomic DNA being present in EOMA CM or playing a role in MF survival, there is still a small possibility that this aspect of LRA treatment may confound our results. Future studies will need to build off these preliminary experiments and determine which lipid species is specifically responsible for prolonging the *in vitro* survival of filarial worms.

Future directions

Future research will focus on the identification of small molecules that support MF survival. This will be accomplished by using mass spectrometry to identify factors between 0.1 – 1 kDa that are present in EOMA CM but absent in RBL-2H3 CM. EOMA CM will first be placed in 0.1 kDa dialysis tubing and dialyzed into ammonium acetate.

This step will remove all molecules smaller than 0.1 kDa, and the supporting factors will be retained in the tube. The sample will then be transferred to 1 kDa dialysis tubing and dialyzed into ammonium acetate with 10-50% acetonitrile. This step will remove all molecules larger than 1 kDa, and the supporting factors between 0.1 – 1 kDa in size will now be present in the buffer outside the dialysis tube. The buffer can then be lyophilized for mass spectrometry analysis. This process will be repeated for RBL-2H3 conditioned media. This media will be used as a negative control to subtract out non-supportive molecules that are produced by RBL-2H3 cells as well as molecules present in basic cell culture media and FBS. Because the samples will contain a complex mixture of peptides and lipids, they will be analyzed using both positive and negative ion mode to ensure that complete coverage is achieved.

Once the molecules that are present only in EOMA CM are identified, they can be supplemented back into DMEM with 10% FBS and assessed for enhanced MF survival. Factors can also be added to larger fractions that did not originally support MF survival. We hypothesize that the addition of these small molecules to the larger fractions will restore MF survival because carrier proteins will be able to bind to and deliver their small molecule ligands.

Although EOMA cells may be directly responsible for producing the nutrients MF require, an alternative hypothesis is that endothelial cells are catalyzing the release of nutrients present in FBS. Lipoprotein lipase (LPL) is expressed on the surface of EOMA cells. This enzyme may play a role in releasing fatty acids and triglycerides from lipoprotein carriers present in FBS, such as chylomicrons and VLDL. Future experiments will test this hypothesis by culturing EOMA cells in the presence of tetrahydrolipstatin

(Orlistat®) or angiopoietin-like 4 (ANGPTL4), which are potent inhibitors of LPL (68; 130). If either inhibitor is toxic to MF, RNA interference may be used as an alternative approach for reducing LPL activity. MF survival will then be tested using EOMA CM obtained from cells treated with LPL inhibitors.

Finally, future work will investigate the role of C3 in MF survival. Because C3 has a short half-life, repeat doses of recombinant mouse C3 will be added to DMEM with 10% FBS to determine if MF survival can be further extended. Additionally, supplementation with C3a or C3b will be tested to determine if a particular component of C3 is more beneficial. Because there are no worm orthologs for human C3 receptors, immunoprecipitation studies will be performed to identify potential filarial complement receptors. Bands that show worm products binding to C3 will be removed and receptors will be identified by mass spectrometry. The identification of worm-specific complement receptors may provide novel drug and/or vaccine targets.

In parallel, we will use the aforementioned methods to determine if the specific factors that support MF also prolong adult worm survival. Because of the vast differences in size and metabolic activity, it is reasonable to assume that adult worms will require much higher concentrations of the supporting factors than MF in order to observe significantly enhanced survival.

CONCLUSION

Our data suggest that filarial infection can be used as a therapeutic agent for type III hypersensitivity, and may have the potential to treat type I hypersensitivity in the setting of prolonged or repeat infection. Furthermore, we provide evidence that infection suppresses chemokine production by eosinophils following FcγRI ligation by immune

complexes. The specific suppression of eosinophil function indicates that infection may be beneficial for eosinophil-mediated diseases, such as asthma and eosinophilic esophagitis. Finally, infection-mediated decreases in autoantibodies and suppression of mesangial cell proliferation signifies that filarial worms have a therapeutic effect on pre-established systemic lupus erythematosus.

Due to the pathogenic nature of filarial infections, use of parasitic antigens in place of live worm infection is highly desired. Mass production of worm antigen and identification of factors eliciting protection against hypersensitivity diseases is hindered by our inability to culture filarial worms *in vitro*. To remedy this, we have developed a culture system to allow for the prolonged survival of filarial worms. In this system, worms are cultured in conditioned media derived from mouse endothelial cells. Endothelial cells release soluble factors in the presence of FBS that are sensitive to heat treatment and lipid depletion. Identification of the specific factors in conditioned media that are responsible for prolonged survival will allow for the production of a chemically defined media and will advance the development of an *in vitro* life cycle.

APPENDIX A

EAR SWELLING IN ANIMALS THAT HAVE BEEN SURGICALLY IMPLANTED WITH ADULT WORMS

BALB/c mice were sensitized to OVA as previously described. Adult *L. sigmodontis* worms were isolated from the pleural cavity of infected jirds (*Meriones unguiculatus*, obtained from TRS Laboratory, Athens, GA). Three adult female worms in RPMI were surgically implanted in the peritoneal cavity of anesthetized mice 2 weeks after OVA sensitization. Control animals received a sham surgery and were injected with an equal volume of RPMI. Three days after implantation, mice were given an intradermal injection of 20 µg OVA in 10 µl of PBS in one ear and 10 µl of vehicle alone (PBS) in the other ear. A micrometer was used to measure baseline ear thickness prior to challenge, and at 1, 2, 12, 24, 48, 72, and 96 hours post challenge. The thickness of the vehicle challenged ear was then subtracted from the thickness of the OVA challenge ear for each animal.

Results

Animals implanted with *L. sigmodontis* adult female worms for 3 days exhibited slightly reduced ear swelling compared to sham animals at late phase time points (Fig 30).

Discussion

Eosinophils are the only cell type to display reduced chemokine production and FcγRI expression in chronically infected animals. We hypothesize that, because only one cell type is affected, worms may be releasing factors that specifically alter eosinophil function. To establish whether alterations in eosinophil function are due to exposure to

worm products or the establishment of immunoregulatory networks as a result of chronic infection, we surgically implanted adult female worms into the peritoneal cavity. Three days later, we performed the ear thickness assay. Preliminary results show a slight reduction in ear thickness following a 3 day exposure to adult worms. This reduction was not significant, however additional animals would need to be tested for more conclusive results. Future experiments utilizing repeat administration of worm antigen or excretory-secretory product in lieu of live worm infection would further confirm whether *L. sigmodontis* antigens can directly suppress eosinophil function.

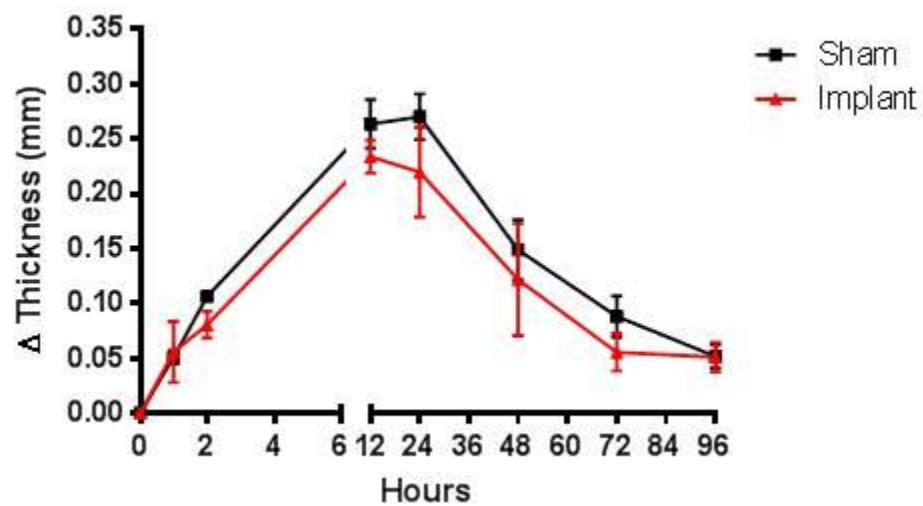


Figure 30. Arthus reaction in animals surgically implanted with adult worms

The ear thickness assay was performed on animals that were given either a sham surgery (black) or had 3 adult female worms implanted in the peritoneal cavity (red). Data represents a single experiment with 7 sham animals and 3 implanted animals.

APPENDIX B

CYTOKINE PRODUCTION BY CD4⁺ T CELLS

Single-cell suspensions of splenocytes were prepared as previously described. Magnetic isolation of CD4⁺ T cells from splenocytes was performed with anti-CD4 mouse microbeads (Miltenyi Biotec, 130-049-201) as per manufacturer instructions. Dendritic cells (DCs) were isolated from splenocytes derived from naïve BALB/c mice with anti-CD11c mouse microbeads (Miltenyi Biotec, 130-052-001). Purified CD4⁺ T cells from experimental animals were cultured at a 10:1 ratio with naïve DCs at a final concentration of 2×10^6 cells/ml. Cells were stimulated with media, 20 µg/ml OVA, 20 µg/ml *L. sigmodontis* antigen (LsAg), or 5 µg/ml anti-CD3 and 2 µg/ml anti-CD28 for 3 days at 37°C and 5% CO₂. Supernatants were collected and stored at -20°C. IL-4, IL-5, IL-10, and IFN-γ ELISAs were performed on cell culture supernatants according to manufacturer instructions (BD Pharmingen).

Results

We sought to determine whether chronic helminth infection altered Th1, Th2 or regulatory cytokine production in response to allergen stimulation. CD4⁺ T cells from Mock, Infected, Sensitized, and Sensitized + Infected animals were cultured with naïve DCs and stimulated with OVA, LsAg, and anti-CD3/CD28. Culture supernatants were then analyzed for the presence of IL-4, IL-5, IFN-γ, and IL-10.

No IL-4 was detectable in response to OVA stimulation for any group of mice, whereas LsAg induced high levels of IL-4 in infected animals (Fig. 31A). All groups had a similar capacity to produce IL-4, as indicated by anti-CD3/CD28 stimulation (Fig. 31B). Sensitized + Infected animals exhibited significantly reduced IL-5 production in

response to OVA stimulation compared to Sensitized animals (Fig. 32A). As expected, LsAg only induced IL-5 production in Infected and Sensitized + Infected animals (Fig. 32B). Infected and Sensitized + Infected animals tended to produce less IL-5 in response to anti-CD3/CD28 stimulation than Mock or Sensitized animals (Fig. 32C), indicating that infection may be suppressing the ability of these mice to mount IL-5-driven responses.

There were no appreciable differences in IFN- γ production in response to OVA stimulation (Fig. 33A), and only infected animals produced IFN- γ in response to stimulation with LsAg (Fig. 33B). Infected and Sensitized + Infected animals had slightly elevated cytokine production in response to anti-CD3/CD28 compared to Mock and Sensitized animals (Fig. 33C).

There were also no appreciable differences in IL-10 response to OVA stimulation (Fig. 34A); however, sensitization prior to infection appeared to suppress the ability of Sensitized + Infected CD4⁺ T cells to produce IL-10 in response to LsAg (Fig. 34B). Nevertheless, Sensitized + Infected animals showed strong IL-10 production in response to anti-CD3/CD28 (Fig. 34C).

Discussion

Overall, there were no major differences in Th1 versus Th2 skewing between groups. Both sensitization and infection induce a Th2 response. Although worm burdens were not altered (Fig. 7), sensitization prior to infection appeared to have a slight effect on cytokine production in response to worm antigen (LsAg). This is not unexpected, as the worms are entering a Th2-skewed environment, rather than the Th1/Th2 neutral environment exhibited by naïve mice. Perhaps the most striking result obtained from the

cytokine assays was the observed suppression of IL-5 production in response to OVA stimulation in Sensitized + Infected animals (Fig. 32A). IL-5 is a Th2 cytokine that mediates eosinophil proliferation, maturation, and survival. This data further supports the hypothesis that chronic filarial infection may be specifically suppressing eosinophil-driven immune responses. Future studies are needed to investigate the mechanism by which worms suppress IL-5 production by CD4⁺ T cells and the importance this has on helminth-mediated suppression of eosinophilic inflammation.

Figure 32. IL-5 production by CD4⁺ T cells

CD4⁺ T cells were isolated from the spleen of Mock, Infected, Sensitized, and Sensitized + Infected animals. CD4⁺ T cells were incubated with DCs from naïve animals and stimulated with OVA (**A**), LsAg (**B**) or anti-CD3/CD28 (**C**). Culture supernatants were then assayed by ELISA to assess IL-5 production.

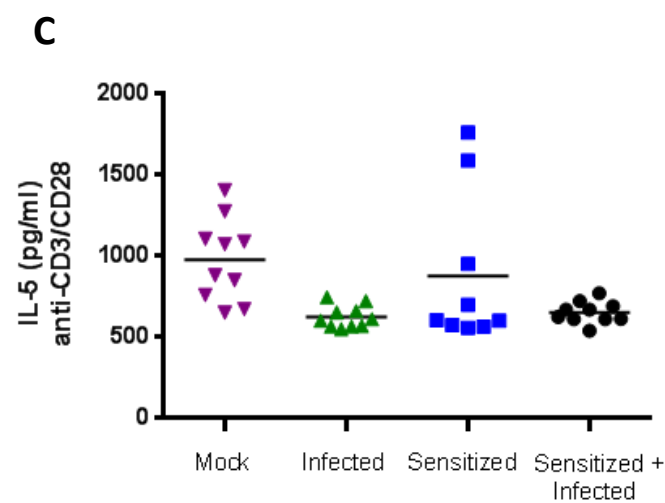
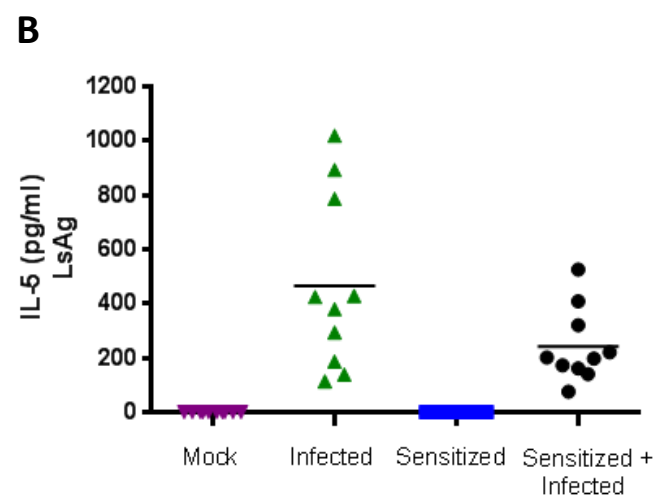
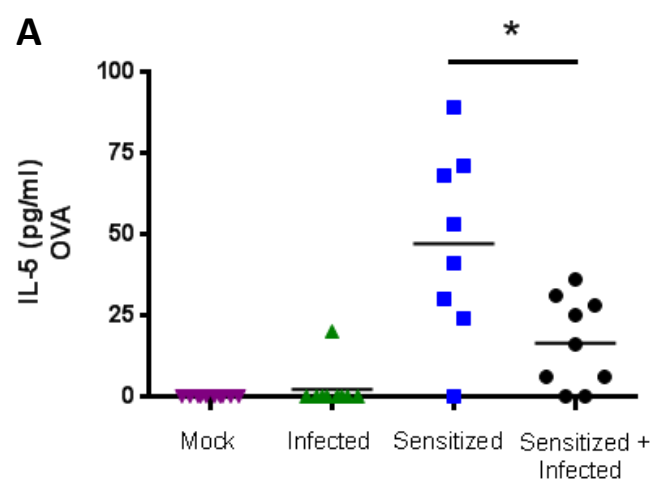
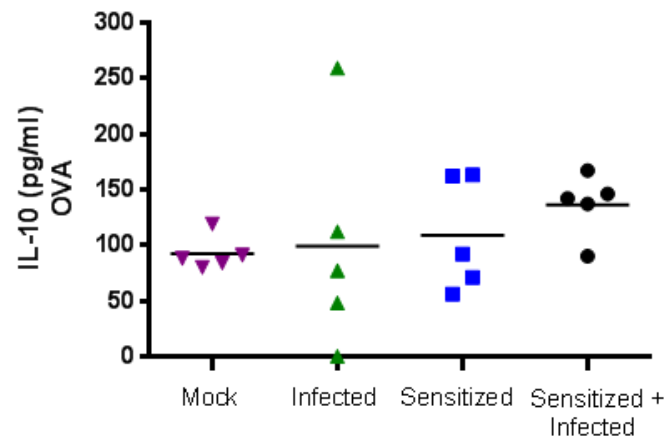
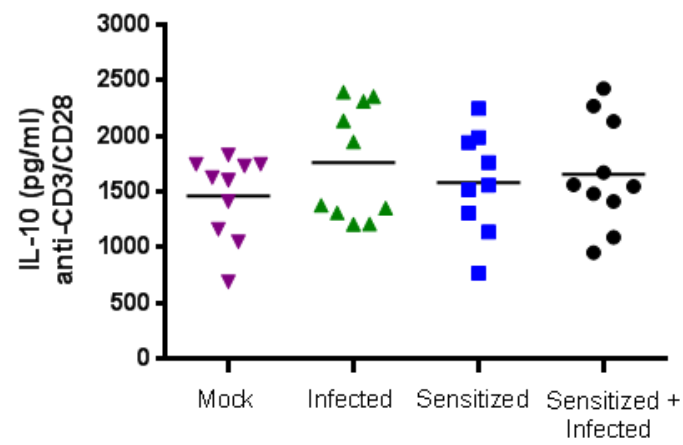


Figure 33. IFN- γ production by CD4⁺ T cells

CD4⁺ T cells were isolated from the spleen of Mock, Infected, Sensitized, and Sensitized + Infected animals. CD4⁺ T cells were incubated with DCs from naïve animals and stimulated with OVA (**A**), LsAg (**B**) or anti-CD3/CD28 (**C**). Culture supernatants were then assayed by ELISA to assess IFN- γ production.

Figure 34. IL-10 production by CD4⁺ T cells

CD4⁺ T cells were isolated from the spleen of Mock, Infected, Sensitized, and Sensitized + Infected animals. CD4⁺ T cells were incubated with DCs from naïve animals and stimulated with OVA (**A**), LsAg (**B**) or anti-CD3/CD28 (**C**). Culture supernatants were then assayed by ELISA to assess IL-10 production.

A**B****C**

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